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# A discussion on The protein molecule

17 November 1938

Opening Address

BY PROFESSOR T. SVEDBERG

The proposal of the subject for this discussion is in itself a remarkable thing and a symbol of the spirit of this meeting. A few years ago the proposal would have looked preposterous. Proteins were known as a mysterious sort of colloids, the molecules of which eluded our search. What is it then that has happened in these years? Why is the most distinguished scientific society of this country inviting a discussion on the protein molecule?

The brilliant work on inorganic colloids, especially on gold sols, by Zsigmondy and others had shown that the mass of the particles of these colloids changed continually with the conditions of their formation. The particles had no individuality from the quantum point of view—therefore they were not molecules although they obeyed the same laws of thermal motion as the molecules. Now the proteins behaved in many respects like inorganic colloids, were held back by membranes, diffused very slowly, etc., and one therefore concluded that the protein particles were not molecules. Another line of thought led to the same conclusion. In spite of all their efforts and the wonderful achievements in other fields the organic chemists were not able to synthesize molecules of a mass approaching—even in a modest way—that of the protein particle. Giant molecules, therefore, were supposed not to exist—only clusters of ordinary small molecules forming particles of undefined mass.

To-day we have, I think, definite proof that this view is wrong. Investigations along different lines have given the result that the proteins are built up of particles possessing the hall-mark of individuality and therefore are in reality giant molecules. We have reason to believe that the particles in



protein solutions and protein crystals are built up according to a plan which makes every atom indispensable for the completion of the structure. The removal of even a single atom means loss of individuality. This is perhaps best seen in the case of proteins containing a prosthetic group. The removal of one atom of iron from cytochrom causes a profound change in the molecule. Protein reactions are therefore elementary acts which must of necessity obey the laws of quantum mechanics.

The evidence for the molecular nature of protein particles and information concerning the mass, shape, structure and chemical properties of the protein molecule has been gained through the application of the following experimental procedures: ultracentrifugal sedimentation, diffusion, osmotic pressure, electrophoresis, X-ray analysis, viscosity, orientation in electric and magnetic fields, stream double refraction, surface films, Tyndall effect, enzymatic and hydrolytic decomposition, kinetics, mutation in viruses and genes.

Among these different modes of attack ultracentrifugal sedimentation and X-ray analysis have perhaps given the most direct proofs of the existence of the protein molecule, such methods of investigation as diffusion, viscosity and stream double refraction have taught us much about the shape of protein molecules, while electrophoresis and decomposition experiments have helped us to collect invaluable information concerning their chemical properties. The X-ray (and possibly the electron-ray) analysis will ultimately give us a complete picture of the architecture of the protein molecule as it exists in crystals. Recent findings seem to indicate that a study of the changes or mutations taking place in giant protein molecules such as we believe are the essence of viruses and the genes of the chromosomes might help us to understand the mode of reproduction or synthesis of the protein molecule.

In ultracentrifugal sedimentation (Svedberg 1925, 1926, 1927, 1938) we measure either the rate of fall of the protein molecule or the equilibrium between fall and diffusion. Especially in the former case a sort of mass analysis is possible. If the solution studied contains a single molecular species or a few kinds of well-defined molecules a diagram showing corresponding discontinuities is obtained. An equilibrium determination on a solution containing only one molecular species gives directly the molecular weight, but in the case of a mixture this method is less efficient. Sedimentation velocity measurements must be supplemented by diffusion determinations in order to give molecular weights.

If we define as sedimentation constant  $s$  the rate of settling in unit centrifugal field and reduced to water of 20° C as solvent we have for the

molecular weight from sedimentation and diffusion (Svedberg 1925, 1927)

$$M_s = \frac{RTs}{D(1 - V\rho)}, \quad (1)$$

where  $R$  is the gas constant,  $T$  the absolute temperature,  $D$  the diffusion constant,  $V$  the partial specific volume of the solute, and  $\rho$  the density of the solution.

Molecular weight from equilibrium data is given by the expression (Svedberg 1925, 1926)

$$M_e = \frac{2RT \ln(c_2/c_1)}{(1 - V\rho) \omega^2(x_2^2 - x_1^2)}, \quad (2)$$

where  $c_2$  and  $c_1$  mean the concentrations at distances  $x_2$  and  $x_1$  from the axis of rotation and  $\omega$  the angular velocity.

In order to ensure sedimentation and diffusion undisturbed by interaction between molecules the concentration of the solute has to be low. It is further necessary to compensate electrical forces (the Donnan effect) by the addition of a low molecular electrolyte (Tiselius 1926, 1932).

The combination of sedimentation and diffusion data means explicit elimination of the frictional constant which is done implicitly in the equilibrium measurements. One might ask whether we are justified in assuming the frictional constant to be the same in sedimentation and diffusion. From a theoretical point of view there is no reason for a difference as long as there is no orientation caused by the sedimentation. In the cases so far studied the rate of fall is so small that any tendency to orientation is completely compensated by the thermal motion (Perrin 1934, 1936). If the centrifugal force varies rapidly per unit of length, as is the case near the axis of rotation, one might expect a certain degree of orientation if the molecules are very elongated. On the other hand, the centrifugal force is low near the axis. For orientation experiments of this kind a very small rotor running at extremely high speed would be best.

The values of specific volume used are based on dry-weight determinations, and the molecular weights accordingly refer to the mass of the molecule without water. Hydration has no influence on the molecular weight values as long as the density of the hydration and adsorption shell around the molecule does not differ from the density of the solution. Even in case of such a difference the error in the molecular weight is very small provided the protein and salt concentration is low (Lausung and Kraemer 1936; Adair and Adair 1936). Needless to say the molecular weight values obtained

by this method are independent of the shape of the molecule and of the validity of Stoke's law.

The molar frictional constant may be calculated from the sedimentation constant  $s$  and the equilibrium molecular weight  $M_e$  or from the diffusion constant by means of the equations (Svedberg 1927)

$$f_s = \frac{M_e(1 - V\rho)}{s}, \quad (3)$$

$$f_d = \frac{RT'}{D}. \quad (4)$$

Experience has shown  $f_s$  to be equal to  $f_d$ .

For a spherical molecule of the same mass the frictional constant is

$$f_0 = 6\pi\eta N \left( \frac{3}{4} \frac{M}{\pi} \frac{V}{N} \right)^{\frac{1}{3}}, \quad (5)$$

where  $\eta$  is the viscosity of the solvent and  $N$  the Avogadro constant.

For a non-solvated spherical particle the experimentally determined frictional constant  $f$  should be equal to  $f_0$ . Deviations of the frictional ratio  $f/f_0$  from unity may therefore be taken as a measure of the deviation from the spherical shape or of hydration or both (Svedberg and Sjögren 1929). If we have reason to believe that there is no solvation and that the dissymmetry of the molecule is roughly that of a stretched ellipsoid of rotation the axial ratio  $a/b$  may be calculated by means of the equation (Gans 1928; Perrin 1934, 1936; Herzog, Illig and Kudar 1934)

$$f_0/f = \frac{1}{2} \left( \frac{(a^2/b^2)^{\frac{1}{2}}}{(1 - a^2/b^2)^{\frac{1}{2}}} \ln \frac{1 + (1 - a^2/b^2)^{\frac{1}{2}}}{1 - (1 - a^2/b^2)^{\frac{1}{2}}} \right). \quad (6)$$

From  $a/b$  and the known volume one finds the dimensions of the molecule.

As an example of sedimentation analysis fig. 1 gives the sedimentation diagram of *Limulus* haemocyanin showing the presence of four homogeneous main components. An equilibrium measurement is exemplified in fig. 2, which demonstrates the molecular homogeneity of phycocerythrin.

Analysis by means of sedimentation velocity measurements has proved that solutions of the native easily soluble proteins are either monodisperse or paucidisperse, i.e. they contain either a single or a few molecular species well defined with regard to mass and shape. Similar although less stringent conclusions can be drawn from sedimentation equilibrium and diffusion measurements. Those proteins, on the other hand, which occur as more or less solid deposits in the organism and are not easily brought into solution—such as keratin, fibroin, myosin—do not form definite molecules.

The application of this technique has further shown that protein molecules are often reversibly dissociated or associated by the action of very mild agents such as change of protein concentration, change of salt concentration, change of *pH*, addition of amino compounds. The products of these reactions

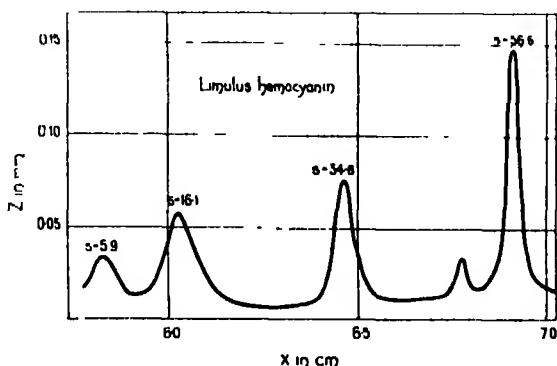


FIG. 1. Sedimentation diagram for *Limulus haemocyanin* obtained by the refractive index method at *pH* 6.8, showing the four main components and also a small amount of a fifth. Centrifugal force 120,000 times gravity. Time after reaching full speed 35 min. (Pedersen.)

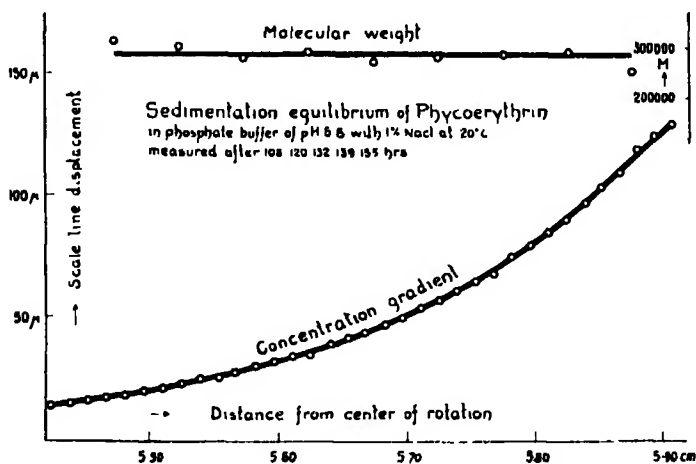


FIG. 2. Relation between molecular weight and distance from centre of rotation for phycoerythrin ( $M = 290,000$ ) at *pH* 6.8. (Eriksson-Quensel.)

have molecular weights connected by a law of simple multiples. The first step in dissociation is mostly a splitting into halves, the first stage in association the doubling of the mass. Thus haemoglobin is split into halves when diluted (Pedersen and Anderson, unpubl.) and upon addition of urea (Steinhardt 1938). Serum albumin is split into fragments probably one-eighth

of the normal molecule by the action of clupein (Pedersen 1936*a, b*, 1937, 1938) (fig. 3). If the pH of a *Helix pomatia* haemocyanin solution is gradually brought from the isoelectric point into more and more alkaline regions the molecules are first split into halves, then into eighths, then into sixteenths (Eriksson-Quensel and Svedberg 1936). Human serum globulin undergoes dissociation and association according to the concentrations of salt and proteins present (Pedersen, unpubl.; McFarlane 1935). In most cases the reaction is reversible.

A third result of ultracentrifugal and diffusion investigations is the finding of a rule of simple multiples for the molecular weights of the proteins. Not

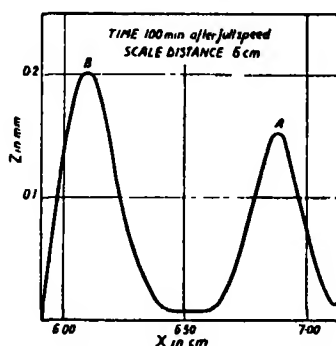


FIG. 3. Sedimentation diagram of serum albumin in 2.6% clupein solution. (Pedersen.)

only nearly related substances such as the red blood pigments obey the rule, but the masses of most proteins, even those of widely different origin, show similar regularities (Table I). If we choose 17,000 as the unit the majority of the proteins may be divided into eleven classes with molecular weights which are multiples of this unit by factors containing powers of 2 and 3. The rule is only approximate, indicating that the underlying principle, which probably means a similarity in the architecture of all proteins, is obscured by some secondary factor. If the proteins are built up of amino-acid residues according to a common plan the differences in percentage of the amino acids of different molecular weight might be regarded as this obscuring factor.

Osmotic measurements may be used for the calculation of molecular weights of proteins (Sørensen 1915-17). The method being in principle a counting of the number of molecules in a known amount of material only mean values of molecular weight are obtained. With increasing molecular weight the accuracy diminishes because of the smallness of the pressures



TABLE I. MOLECULAR CONSTANTS OF PROTEINS

Protein	$s_0$	$D_0$	$M_r$	$M_v$	$M_{calc}$	$f/f_0$	Isoclectric point	$\frac{d\mu}{d\ln H_2} \times 10^4$	Authorities
Erythrocyte (Lampyris)	1.87	10.45	17,100	16,100	17,000	1.2	5.40	5.2	Pedersen (unpubl.); Polson (unpubl.); Svedberg and Eriksson-Quensel (1934)
Lactalbumin A	1.9	10.6	17,600	—	—	1.8	5.12	6.7	Pedersen (1936a); Polson (unpubl.)
Cytochrome c	1.89	10.13	16,600	—	—	1.3	5.7	—	Pedersen and Anderson (unpubl.); Theorell (1935)
Myoglobin	2.04	11.32	17,200	17,600	—	1.1	7.0	7.0	Polson (unpubl.); Theorell (1935)
Bacillus Pasteur protein	1.8	10.2	17,000	—	—	1.2	—	—	Seibert, Pedersen and Tiselius (1938)
Gladin	2.0	6.72	26,000	—	—	1.6	—	—	Arborelius (1937); Polson (unpubl.)
Hordelin	2.0	6.5	27,000	—	—	—	—	—	Quensel and Svedberg (1938)
Zellin	1.9	4.0	26,000	—	35,200 = 2 x 17,000	—	—	—	Watson, Arborelius and Williams (1936)
Erythrocyte (Aren)	2.42	—	—	35,000	—	1.0	—	—	Svedberg and Eriksson-Quensel (1934)
Erythrocyte (Chironomus)	2.00	—	—	31,400	—	1.6	6.40	3.6	Pedersen (unpubl.); Svedberg and Eriksson-Quensel (1934)
Lactoglobulin	2.12	7.27	41,400	37,000	—	1.2	5.19	11.0	Pedersen (1936a); Polson (unpubl.)
Pepsin	3.3	9.00	35,500	36,200	—	1.1	—	—	Eriksson-Quensel (unpubl.); Philpot (1935); Polson (unpubl.)
Insulin	2.47	8.20	40,000	36,100	—	1.1	—	—	Sjögren and Svedberg (1931)
Bence-Jones α	2.65	—	—	35,000	—	1.0	6.20	5.8	Svedberg and Sjögren (1935); Tiselius (1936)
Bence-Jones β	2.85	7.33	27,700	—	—	1.3	5.48	3.6	Pedersen and Anderson (unpubl.); Polson (unpubl.)
Waldmann	2.65	7.75	42,500	40,600	—	1.1	4.55	10.4	Larum and Polson (1936); Pedersen (unpubl.)
Human subunit: Bacillus protein	2.3	8.2	22,000	—	—	1.2	4.3	—	Seibert, Pedersen and Tiselius (1938)
Concanavalin B	2.49	7.4	42,000	—	—	1.3	—	—	Sumner, Grålin and Eriksson-Quensel (1936a)
Orotin	2.14	8.6	26,000	—	—	1.2	—	—	Grålin and Svedberg (1938)
CO-haemoglobin (horse)	4.5	6.3	64,000	68,000	70,400 = 4 x 17,600	1.2	6.22	7.2	Svedberg and Hållén (1930); Tiselius and Gross (1934)
CO-haemoglobin (man)	4.5	6.9	65,000	—	—	1.3	7.08	6.4	Larum and Polson (1936); Pedersen (unpubl.)
Serum albumin (horse)	4.5	6.17	70,200	66,800	—	1.2	4.80	9.1	v. Mutzenbecher (1933); Pedersen (unpubl.); Polson (unpubl.)
Yellow ferment	5.76	6.28	82,800	77,800	—	1.2	5.22	6.4	Kokwick and Pedersen (1936); Polson (unpubl.)
Concanavalin A	6.0	5.6	98,000	—	106,000 = 6 x 17,600	1.1	—	—	Sumner, Grålin and Eriksson-Quensel (1936a)
Orovalin	6.4	5.1	113,000	—	—	1.3	—	—	Sumner, Grålin and Eriksson-Quensel (1936a)
Serum globulin (horse)	7.1	4.05	167,000	150,000	140,800 = 8 x 17,600	1.4	$\alpha f = 5.1$ $\gamma = 9.0$	—	Eriksson-Quensel (unpubl.); v. Mutzenbecher (1933); Tiselius (1937a)
Antipneumococcus serum globulin (rabbit, monkey)	6.9	4.14	107,000	—	—	1.4	Rabbit 5.8 Monkey 5.8	—	Kabat and Pedersen (1938); Tiselius and Kabat (1938)
Antipneumococcus serum globulin (man)	7.4	3.90	185,000	—	—	1.5	—	—	Kabat (1939)
Phycocyanin (Cocconeis, dissociation component)	6.8	4.58	131,000	146,000	—	1.4	4.85	10.2	Eriksson-Quensel (1938); Pedersen (unpubl.); Polson (unpubl.)
Phycocyanin (Cocconeis)	12.0	4.00	220,000	222,000	222,000 = 16 x 17,600	1.2	4.25	14.2	Eriksson-Quensel (1938); Tiselius (1936); Tiselius and Gross (1934)
Physocyanin (Cocconeis, main component)	11.4	4.05	272,000	272,000	—	1.2	4.85	10.2	Eriksson-Quensel (1938); Pedersen (unpubl.); Tiselius and Gross (1934)
Edestin	12.0	3.93	309,000	—	—	1.2	—	—	Polson (unpubl.)
Bovulin	12.8	4.06	294,000	—	—	1.1	—	—	Polson (unpubl.)
Amazulin	12.0	3.68	329,000	—	—	1.3	—	—	Polson (unpubl.)
Catalase	11.3	4.1	248,000	—	—	1.3	—	—	Sumner and Grålin (1938)
Serum globulin (Lampyris)	12.0	3.2	358,000	—	—	—	—	—	Svedberg and Anderson (1938) and unpubl.
Erythrocyte (Daphnia)	16.3	—	—	—	422,000 = 24 x 17,600	—	—	—	Svedberg and Eriksson-Quensel (1934)
Haemocyanin (Pandalus)	17.4	—	—	397,000	—	1.1	—	—	Eriksson-Quensel and Svedberg (1936)
Haemocyanin (Pandalus)	16.4	2.4	445,000	447,000	—	1.2	—	—	Eriksson-Quensel and Svedberg (1936); Polson (unpubl.)
Haemocyanin (Hélix pomatia, dissociation component)	12.1	2.23	502,000	—	—	1.5	6.08	8.1	Eriksson-Quensel and Svedberg (1936); Polson (unpubl.); Tiselius (1936)
Haemocyanin (Bryozoa, dissociation component)	13.5	2.29	378,000	—	—	1.4	4.40	10.7	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.); Polson (unpubl.)
Haemocyanin (Hélix pomatia, dissociation component)	10.9	2.25	440,000	—	—	1.9	4.4	14	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.); Polson (unpubl.)
Urease	18.6	2.46	483,000	—	—	1.2	—	—	Sumner, Grålin and Eriksson-Quensel (1936a)
Thyroglobulin (pig)	19.2	2.65	628,000	650,000	—	1.6	4.58	11	Heidelberger and Pedersen (1935); Polson (unpubl.)
Haemocyanin (Nephele)	24.5	2.70	820,000	—	846,000 = 48 x 17,600	1.3	4.44	13.3	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.); Polson (unpubl.)
Haemocyanin (Homarus)	22.6	2.78	752,000	805,000	—	1.3	4.66	18	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.); Polson (unpubl.)
Haemocyanin (Hélix pomatia, dissociation component)	16.0	1.83	814,000	797,000	—	1.9	5.06	8.1	Eriksson-Quensel and Svedberg (1936); Pedersen (1933); Polson (unpubl.)
Haemocyanin (Hélix pomatia, dissociation component)	16.6	1.92	790,000	—	—	1.8	4.83	11.4	Eriksson-Quensel and Svedberg (1936); Pedersen (1933); Polson (unpubl.)
Antipneumococcus serum globulin (horse, cow, pig)	18.0	1.85	920,000	—	—	2.0	Horse 4.4 Cow 4.6 Fig. 6-1	—	Kabat and Pedersen (1938); Tiselius and Kabat (1938)
Erythrocyte (Planorbis)	23.7	1.96	1,686,000	1,530,000	1,900,000 = 96 x 17,600	1.4	6.77	10.6	Larum and Polson (1936); Pedersen (1933); Svedberg and Eriksson-Quensel (1934)
Haemocyanin (Cyclops)	24.0	—	—	1,329,000	—	1.2	—	—	Eriksson-Quensel and Svedberg (1936); Svedberg and Hedström (1934)
Haemocyanin (Cyclops)	49.2	1.65	2,786,000	—	—	1.4	—	—	Eriksson-Quensel and Svedberg (1936); Polson (1936)
Haemocyanin (Eidonia)	49.1	1.64	2,790,000	—	—	1.4	4.6	14	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.); Polson (unpubl.)
Erythrocyte (Arenicola)	67.4	—	—	3,000,000	3,380,000 = 192 x 17,600	1.0	4.84	18	Pedersen (1933); Svedberg and Eriksson-Quensel (1933)
Olochromycin (Spirographis)	66.2	—	—	—	—	—	—	—	Eriksson-Quensel (unpubl.)
Haemocyanin (Rana)	66.2	1.58	3,316,000	—	—	1.4	—	—	Eriksson-Quensel and Svedberg (1936); Polson (unpubl.)
Erythrocyte (Lumbricus)	60.9	1.81	3,140,000	2,844,000	—	1.3	5.28	12.6	Pedersen (unpubl.); Polson (unpubl.); Svedberg and Eriksson-Quensel (1933)
Haemocyanin (Hélix pomatia, main component)	66.9	1.38	6,680,000	6,680,000	6,760,000 = 384 x 17,600	1.3	6.05	8.1	Eriksson-Quensel and Svedberg (1936); Polson (unpubl.); Tiselius (1936)
Haemocyanin (Bryozoa, main component)	101.7	—	—	—	—	1.3	4.48	10.7	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.)
Haemocyanin (Bryozoa, aggregation component)	130.4	—	—	—	—	1.6	4.46	10.7	Eriksson-Quensel and Svedberg (1936); Pedersen (unpubl.)





observed. Donnan effects have to be corrected for or eliminated by the addition of salt. Theory and experimental technique were worked out by Adair (1928, 1935), and a number of reliable values have been obtained by him (Adair 1925, 1938; Adair and Robinson 1930; Adair and Roche 1934).

From measurements of the migration of protein molecules in an electric field—electrophoresis (Svedberg and Tiselius 1926; Tiselius 1930)—we cannot, by elimination of the frictional constant, arrive at values for the electric charge. Electrophoresis data are, however, of great importance for the characterization of the proteins. The position of the isoelectric point and the slope of the mobility curve reflect to a certain degree the composition of the molecule and may serve as an index of its chemistry (Table I). Slight changes in the structure not revealed by a mass analysis (ultracentrifugal sedimentation) will show up in electrophoresis. A greatly improved technique (Tiselius 1937*b*) has recently made possible important investigations in this field.

Among the results of electrophoretic measurements the demonstration of species differentiation in blood pigments of identical molecular weight may be mentioned. Thus the isoelectric points of the haemocyanins of *Helix pomatia* and *H. nemoralis* with the same molecular weight, 6,700,000, are 5.05 and 4.63 (Pedersen 1933). Electrophoretic determinations have made it possible to prove intercombination of the dissociation products when solutions of these two haemocyanins are mixed at a *pH* where they are split and then brought to a *pH* low enough to cause recombination (Tiselius and Horsfall 1939).

The application of the new electrophoresis technique to the study of serum proteins has yielded important results (Tiselius 1937*a*). Thus in normal mammalian serum three electrochemically well-defined globulins now called  $\alpha$ ,  $\beta$  and  $\gamma$  were found. These proteins have approximately the same molecular weight in dilute solution but quite different electrochemical properties, e.g. the isoelectric point of globulin  $\gamma$  is at *pH* 6.0 instead of 5.1, as for the  $\alpha$  and  $\beta$  components. In addition the mobilities are quite different, especially in the alkaline region. A separation by means of electrophoresis is therefore comparatively easy. The study of immune sera has shown that the antibody activity is in some cases connected with the  $\gamma$ -component, while there sometimes appears a new component as the carrier of the activity (Tiselius 1937*a*, 1937*c*; Tiselius and Kabat 1938). This proves the high sensitivity of electrochemical behaviour to changes in molecular structure.

X-ray analysis of protein crystals and semi-solid protein deposits in living organisms has yielded results of the highest importance for the

elucidation of the structure of the protein molecule. Investigations by the two British schools (Bernal and others 1938; Crowfoot 1938; Astbury 1938) have shown that the proteins may be divided into two classes, globular proteins and fibrous proteins. In the latter case the structure is that of a polypeptide chain grid, while in the former case a multiple folding obtains resulting in a high degree of symmetry of crystals of globular proteins. Denaturation of this type of proteins would correspond to an unfolding and accordingly to the appearance of less folded polypeptide chains. A more detailed study has brought to light certain facts, such as the existence of long spacings in the structure of the fibrous proteins, tending to make the dividing line between the two groups less distinct. Astbury seems to be of the opinion

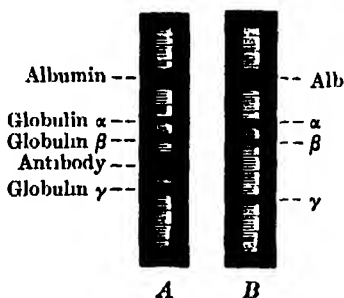


FIG. 4. Electrophoretic diagrams of unadsorbed (*A*) and adsorbed (*B*) antipneumococcus horse serum. (Tiselius.)

that after all there may be macro-molecules even in the protein fibre (Astbury 1937).

Molecular weights of globular proteins calculated from X-ray data agree very well with the values obtained from ultracentrifugal determinations. Thus the Bernal school found for pepsin, insulin, lactoglobulin and haemoglobin the values 40,000, 37,200, 36,500, 69,000 respectively, while the ultracentrifuge gives 37,000, 38,000, 39,000 and 68,000 respectively (Bernal and Crowfoot 1934; Bernal and others 1938; Crowfoot 1935, 1938). X-ray analysis proves the molecular homogeneity of the globular proteins in the crystals. An interesting result is the high degree of symmetry displayed by the protein lattice. Haemoglobin has a face-centred pseudo-hexagonal elementary cell and the tobacco seed globulin a face-centred pseudo-cubic cell (Bernal and others 1938). The length of the haemoglobin molecule in wet crystals is estimated to 109 Å and the breadth to 32 Å. The frictional ratio  $f/f_0$  as determined by means of ultracentrifugal sedimentation and diffusion is 1.2. Under the assumption of an ellipsoidal shape we get for the

axial ratio of the dehydrated molecule 0.27, while the X-ray data require about 0.29.

An interesting attempt to construct a three-dimensional model of the protein molecule out of a few assumptions and purely geometrical reasoning has been done by Dr Dorothy Wrinch, Oxford (1937*a*, 1938). A certain transfer of bonds is assumed, resulting in plane triangular groups which can be linked together to a more or less complicated "cyclol" pattern. It is further shown by Miss Wrinch that there exist certain polyhedrons on the surface of which a "cyclol" network can be drawn. The truncated tetrahedron seems the most likely type. A closed "cyclol" network can be fitted on its surface fulfilling the condition that the faces form the tetrahedral angle at the edges where the "cyclol" network crosses over. A linear series of such tetrahedron networks comprising 72, 288, ...,  $72n^2$  amino-acid residues may possibly represent the different molecular weight classes of the proteins. The cyclol with 288 amino-acid residues has been studied in detail (Wrinch 1937*b*; Langmuir and Wrinch 1938) and its properties compared with the known properties of insulin—a protein investigated both by means of chemical analysis, X-rays and ultracentrifugal sedimentation.

Viscosity measurements may be used for the estimation of the dimensions of protein molecules. The theories which have been developed for the description of viscosity in terms of molecular dissymmetry are, however, not very clear and certain assumptions have to be made. For rod-shaped molecules Kuhn (1932) has proposed the relationship

$$\frac{\eta - \eta_0}{\eta_0} = 2.5G + \frac{G}{16} \left( \frac{b}{a} \right)^2, \quad (7)$$

where  $\eta_0$  is the viscosity of the solvent,  $G$  is the volume of solute per c.c. and  $b/a$  is the ratio of the long to short axis of the molecule. According to recent deductions by Burgers (1938) we have for an elongated ellipsoid of rotation

$$\frac{\eta - \eta_0}{\eta_0} = Gk, \quad (8)$$

where  $k$  is a certain function of the axial ratio of the molecule.

Polson (1936) has studied the relation between viscosity and axial ratio for various proteins using axial ratios calculated from values of  $f/f_0$  according to equation (6). It was found that the empirical formula

$$\frac{\eta - \eta_0}{\eta_0} = 4.0G + 0.098G \left( \frac{b}{a} \right)^2, \quad (9)$$

describes the behaviour of all the proteins so far investigated. This shows that the relationship between viscosity and molecular shape is about the

balance method to proteins by E. Gorter (1937) and collaborators (Philippi 1936) and by Rideal (Hughes and Rideal 1932; Schulman and Rideal 1933; Mitchell 1937) gave results which at first appeared difficult to interpret and seemed to be in conflict with the findings of the ultracentrifugal and X-ray technique. The chief difficulty was the low values for the thickness of the films found which indicated either smaller molecular diameters—admitting globular shape—or very asymmetrical molecules. Neither of these possibilities are compatible with the state of protein molecules in solutions. After Langmuir, Schaefer and Wrinch (1937) had shown that such films could be transferred to solid surfaces and that piles of monolayers could be built up capable of being handled as ordinary membranes, results of importance for the understanding of the structure of the protein molecule were arrived at. Astbury and co-workers (1938) have made X-ray studies of these protein membranes and have been able to measure directly the thickness of bundles of a known number of monolayers. For egg albumin a value of 9–10 Å was found in agreement with X-ray values for the side-chain spacing of fibrous proteins. The diameter of the egg-albumin molecule being of the order of 40 Å it is certain, therefore, that the protein molecule is deformed or even denatured to a considerable degree in surface films.

The most promising method for the elucidation of molecular shape based on optical measurements of orientation is the study of stream double refraction. Work by Boeder (1932), Kuhn (1933) and Haller (1932) has clarified the theoretical side of the problem. The experimental investigations are just beginning to give results. Von Muralst and Edsall (1930) studied myosin, and Boehm and Signer (1931) ovalbumin, myogen, gelatin, myosin and ovoglobulin. The measurements indicate thread-like molecules in the case of myosin and ovoglobulin and practically spherical molecules for egg albumin and myogen. Recent determinations in Upsala by Snellman and Björnsthåhl have shown that in certain antibodies the molecules are very elongated (Snellman and Björnsthåhl, unpubl.).

Studies of the action of enzymes on proteins may serve as a means for getting information about the structure of the protein molecule. Ultracentrifugal and electrophoresis measurements on the cleavage products seem of special interest. Among the attempts made (Annetts 1936; Tiselius, unpubl.) those of Tiselius concerning the action of pepsin on egg albumin merit attention. Using his new electrophoresis method he found that the decomposition product has a much lower mobility than the unchanged protein (fig. 5). Ultracentrifugal sedimentation determinations showed that the molecular weight is low, of the order of peptides.

Speculations on the constitution of the protein molecule based upon the

amino-acid composition have recently been brought into the focus of interest by Bergmann and Niemann (1937*a, b*). Complete knowledge of the content of the various amino acids is not available, but from the data collected through chemical analysis of the products of incomplete hydrolysis certain conclusions may be drawn. Following up an idea first expressed by Astbury (1934), Bergmann and Niemann postulate a general law which expresses the constitution of the proteins as a function of the "frequencies" of the various



FIG. 5. Electrophoretic diagrams of unchanged egg albumin (upper pictures) and egg albumin acted upon by pepsin (lower pictures). (Tiselius.)

amino acids. Each amino-acid residue is supposed to occur at regular intervals in the polypeptide chain. The one with the highest frequency  $F_i$ , or lowest absolute number  $N_i$ , according to the expression

$$F_i N_i = N_t, \quad (13)$$

where  $N_t$  is the total number of amino-acid residues, determines the minimum molecular weight. The frequency values  $F_i$  deduced by Bergmann and Niemann from the analytical data available are very nearly multiples of powers of 2 and 3, and so are the  $N_i$ . The molecular weights deduced by Bergmann and Niemann agree very well with those found by means of ultracentrifugal sedimentation. The classification of the proteins according to molecular weight attempted on the basis of ultracentrifugal data may be explained by the law postulated by Bergmann and Niemann.

In emphasizing the importance of the generalizations just discussed, especially as a stimulus for further research, one should not overlook certain serious difficulties. As pointed out by Astbury (1937), the frequencies calculated by Bergmann and Niemann would in many cases require that one and the same place in the polypeptide chain be occupied by two or more different amino-acid residues. Furthermore, the scheme is not easily compatible with the general structure of proteins established by X-ray measurements. The analytical data available at present, especially those relating to the amino acids of low percentage, are rather uncertain. Now  $N_t$  and therefore the molecular weight depends on the knowledge of the content of these rarer acids. As an example the deductions by Bergmann and Niemann concerning haemoglobin may be mentioned. Here from the cystein content, 0.5 %, they calculate  $N_t = 3$  for this amino acid and find the total number of residues in the molecule to be 576. Their molecular weight value is 69,000, in excellent agreement with ultracentrifugal and osmotic values. Now the ultracentrifugal investigations have shown that haemoglobin can split reversibly into half molecules and that the two halves are probably equal (Steinhardt 1938). X-ray measurements indicate the same (Bernal and others 1938; Crowfoot 1938). Bergmann and Niemann ought therefore to have found a minimum weight of about 34,500 instead of 69,000 for haemoglobin.

The study of chemical reactions in protein solutions by means of physico-chemical methods will probably yield valuable information concerning the structure of the protein molecule. The exceptionally rapid increase of reaction velocity (denaturation) with temperature and with change in  $pH$  which is characteristic of the proteins has been a great puzzle. Activation energies, calculated from temperature coefficients in the usual way, give abnormally high values. A promising attack on the problem has recently been made by Steinhardt (1937), who studied in detail the  $pH$  and temperature inactivation of pepsin. From his measurements he concludes that the rate of reaction depends on the concentration of one particular kind of pepsin ion, and that the amount of this ion varies rapidly with  $pH$  and with temperature. Taking this into account he arrives at reasonable activation energies. The very rapid increase of ions of the sensitive type with  $pH$  explains the sharp  $pH$  stability limits found for the proteins by means of ultracentrifugal and diffusion measurements.

The mechanism of protein synthesis is practically unknown. Recently some interesting indications as to the mode of reproduction of a certain protein have been observed in the field of viruses and genes (Bergmann and Niemann 1937*b*; Gulick 1938; Jordan 1938; Stanley 1938). The fact that

several viruses are now obtained in crystalline form and shown to possess all the properties of proteins of very high molecular weight makes it almost certain that they are to be regarded as protein molecules and not as living beings (Bawden and Pirie 1938; McFarlane and Kekwick 1938; Stanley 1938). On the other hand, the viruses propagate only in living tissues, and it has been suggested that the multiplication is due to an act of autocatalysis, a sort of enzymatic reaction where the virus molecule acts as its own enzyme. The spontaneous mutations observed in plant viruses are thought to be the result of the multiplication of low percentage by-products from the protein synthesis. A number of facts point in the direction of the genes being large individual protein molecules. The propagation of the gene is according to this view regarded as an autocatalysis as in the case of the viruses. The mutations caused by radiation would mean that the gene molecule has been changed by the absorption of a quantum and that this change is being reproduced autocatalytically.

In the above survey I have aimed at showing what means are used at present and might with success be used in the near future for the study of the protein molecule. I have also tried to give a summary of our present knowledge of its main properties. We have still a long way to go before reaching our goal—the complete elucidation of the architecture of the protein molecule. But looking back on the stretch already traversed we may feel justified in stating that the application of physico-chemical methods to the protein problem has already given us much valuable information. The vague term “colloid” for a protein has been replaced by precise information concerning the mass and electrochemical behaviour and in certain cases even of the shape and structure of the protein particles in solutions and crystals. These entities appear so well defined that we really feel justified in denoting them as protein molecules.

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Dr K. LINDERSTRÖM-LANG. *Globular proteins and proteolytic enzymes.*

Genuine globular proteins are slowly attacked by crystalline trypsin under conditions (pH, temperature) where the proteins are stable in the

absence of trypsin. On the other hand, crystalline trypsin is able to split synthetic peptides. The deduction from this that peptide bonds are present in genuine globular proteins would lose its validity in the following case.

If, according to Anson and Mirsky, denaturation is reversible, then in a solution of a given globular protein there is an equilibrium between genuine and denatured protein,



Hence it is sufficient that  $D$  and only  $D$  should contain peptide bonds open to fission by trypsin, because by removal of  $D$  by hydrolysis this process is forced in the direction from left to right and  $G$  will gradually disappear as well. The problem is open to experimental test in the following way.

When the protein is hydrolysed with so much trypsin that the rate of the above process becomes the limiting factor in the total reaction, then we must expect the temperature coefficient for the hydrolysis to approach that of the reversible denaturation, which is presumably very high.

If a protein solution is heated for a short time to a temperature at which the process is proceeding rapidly and completely from left to right and then quickly cooled down to a temperature where it is a slow process, a protein solution is obtained which contains initially more  $D$  than corresponds to the equilibrium at that (low) temperature. Hence we may expect to find a more normal (that is, a lower) temperature coefficient for the hydrolysis of the protein in this solution. Upon standing, the equilibrium will slowly be reached and the temperature coefficient of the hydrolysis by much trypsin will tend to rise correspondingly.

Some preliminary experiments have shown this and seem to provide sufficient basis for giving a warning against the conclusion that genuine proteins contain peptide bonds because they are split by proteinases like trypsin. They give a certain indication that peptide bonds are formed or "appear" (like SH-groups) upon denaturation, but they are not conclusive enough to decide whether or not some hydrolysable peptide bonds are preformed in the molecules of the genuine globular proteins.

G. S. ADAIR. *The size and charge of protein molecules.*

Svedberg and Eriksson-Quensel (1935-6) have shown that the observed values of the rates of sedimentation and diffusion of proteins are lower than the values calculated for anhydrous spherical molecules. This reduction is usually attributed to deviations from the spherical form. An alternative

hypothesis, that in certain cases, including albumin and haemoglobin, the size of the molecules is increased by hydration, is supported by the calculations recorded in Table I.

TABLE I

Proteins	Mol. wt anhydrous	Mol. wt. hydrate	Crystal density	Minimum radius crystal	Maximum radius diffusion
Egg albumin	43,800	58,000	1.239	26.4	27.5
Serum albumin	72,000	96,750	1.237	31.4	33.1
Haemoglobin	67,000	90,050	1.225	30.7	30.9

The third column gives the molecular weights of the proteins, including the water of hydration calculated from measurements of the densities of protein crystals when suspended in concentrated sodium phosphate mixtures (Adair and Adair 1936). The fifth column gives the radius of the molecules in Angstrom units, calculated from the molecular weight and density of the hydrate. These figures represent the minimum values, because it is possible that, in addition to water, the protein may take up a certain amount of the dispersion medium, and it is not unlikely that the hydration of the protein is greater in dilute than in concentrated salt solutions.

The sixth column gives the maximum value of the radius calculated from the measurements of diffusion made by Lamm and Polson (1936) on the assumption that the molecule is spherical. It will be seen that the agreement between the minimum and the maximum values is fairly close.

The important development of methods for the cataphoresis of proteins, due to Tiselius (1937) has rendered it possible to correlate the mobilities of protein ions with their electric charges, calculated from membrane potentials (Adair and Adair 1934). Both methods show that in the presence of increasing concentrations of phosphate ions, the isoelectric point of haemoglobin moves towards the acid side.

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Dr K. O. PEDERSEN. *Interaction of proteins in solution.*

According to ultracentrifuge experiments the "globular" proteins often exhibit association and dissociation reactions when the  $pH$  of the solution is changed (Svedberg and co-workers), the salt concentration is altered (Lundgren, Pedersen) or a protein of lower molecular weight is added (Pedersen 1938). In the last type of reaction the chemical nature of the interacting proteins seems to be important. Some proteins give rise to a large effect, others only to a small one. The amount of dissociation products increases with increasing concentration of the low molecular protein. If such a solution, showing a large effect, is diluted, the normal size molecules are re-formed. It is possible, however, by means of the ultracentrifuge separation cell, to demonstrate the presence of the dissociation products and to isolate these in the upper compartment of the separation cell. These products may also be kept in *dilute solutions*. In some cases it is, however, necessary to add only a small amount of the material from the lower compartment in order to get the normal molecules formed. The lower compartment probably contains either some substance which is necessary for the construction of the normal molecules or some molecules which act as a pattern for the production of the large molecule.

For the protein molecules which exhibit interaction phenomena and whose molecular weights fit into the Svedberg system of multiples the following working hypothesis was sketched. These protein molecules have a primary and a secondary structure. The primary structure is the polypeptide chain which may perhaps be arranged in cyclol cages (Wrinch) or in some other "unit" which form the bricks of the building. The bricks are kept together by means of a non-protein cement which plays a fundamental rôle in the interaction phenomena. In some of the proteins the bindings are made by means of carbohydrates, in others by phosphatides (perhaps through salt-linkages) or by nucleic acids. These substances are responsible for the secondary structure of the protein molecules. Many such substances may, however, be adsorbed on a protein molecule without having anything to do with the construction of the molecule. They may be removed without seriously changing the structure of the protein molecule. Often these adsorbed low molecular substances may, however, act as a plaster and protect the cement and the bricks from being destroyed.

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F. J. PHILPOT AND J. ST L. PHILPOT (with technical assistance by E. DODWELL). *The effect on calcium on the sedimentation constant of casein.*

In attempting to precipitate casein from milk we found that its salting-out properties were markedly altered when the calcium was removed by oxalate. We therefore examined oxalated milk in the ultracentrifuge and found that the casein formed a perfectly homogeneous component of sedimentation constant 6. In view of Pedersen's finding (1936) that in milk dialysed against phosphate buffer the casein had sedimentation constants of 10 and upwards, although Svedberg and others (1930) had previously found

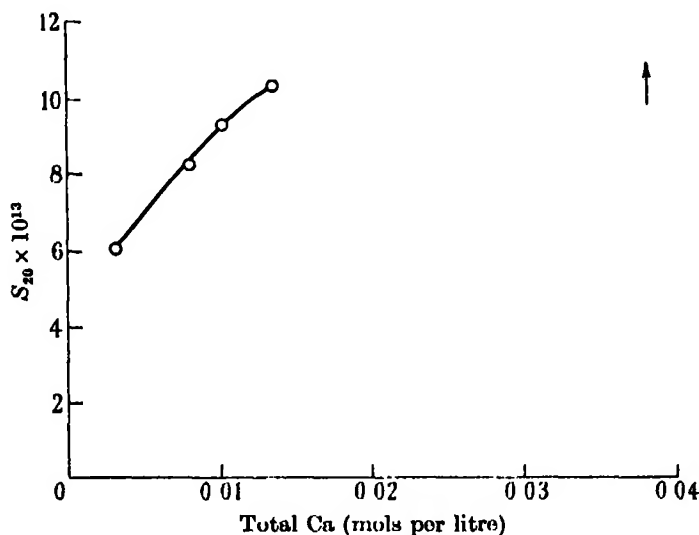


FIG. 1

6 for acid-precipitated casein, we added small amounts of calcium and found that we could produce any desired sedimentation constant up to 10.4, above which coarse particles similar to those in the original milk were formed. Fig. 1 shows the sedimentation constant plotted against the total calcium concentration. This seems to be a good example of a type of sedimentation predicted by Pedersen, where two molecular sizes are in such mobile equilibrium that they sediment as a single component at an intermediate rate. The intermediate mixtures might be expected to appear inhomogeneous; but fig. 2 shows that there is no sign of this.

The precipitate obtained by addition of 16%  $\text{Na}_2\text{SO}_4$  to oxalated milk, when washed and redissolved, consists of homogeneous casein with very little whey protein. Since even oxalated milk still contains some residual calcium, which may not be equally divided between precipitate and

mother liquor, the two fractions may have different sedimentation constants. In one case the precipitate had sedimentation constant 7.8, the mother liquor 9.7, and the mixture of the two 8.7, and fig. 3 shows that in all three solutions the casein part is quite homogeneous.

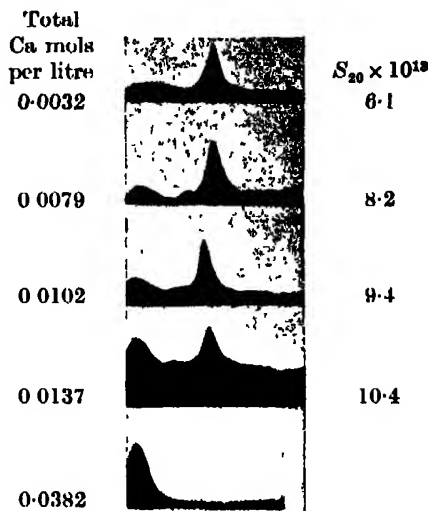


FIG. 2

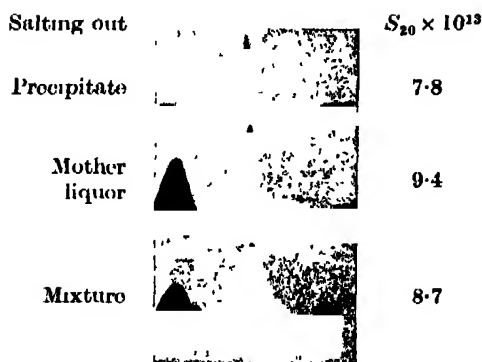


FIG. 3

We took the opportunity to study the first stage of the clotting process. Oxalated milk at pH 7.0, treated with pepsin, showed slight inhomogeneity in the casein, but no appreciable change in sedimentation constant.

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J. ST L. PHILPOT AND P. A. SMALL. *A clue to the structure of pepsin.*

Herriott (1937) showed that pepsin was inactivated by iodine, and he regarded this as due to substitution in the tyrosine groups. We have used Folin's phenol reagent to measure the extent of substitution of the tyrosine groups, and we have found that at pH 5.4 some other groups react with iodine or hypiodous acid more rapidly than the tyrosine groups. Further, when the other groups have reacted the pepsin is inactive. This is shown in fig. 1, where the percentage of activity and of unsubstituted tyrosine are plotted against the amount of added hypiodous acid. At present we have

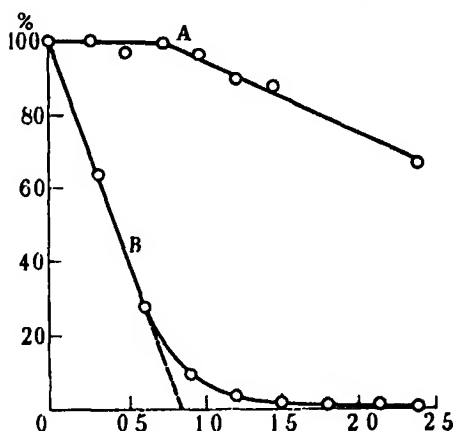


FIG. 1. A, Unsubstituted tyrosine; B, Peptic activity.

no idea what the unknown groups are, but they are not tryptophane, histidine, proline, hydroxyproline, sulphhydryl, amino, carboxyl, peptide, simple ketone, or simple phenol. We therefore think that they may consist of some unusual substance peculiar to pepsin.

We have tried to detach the unknown substance from pepsin by non-hydrolytic procedures in the hope that it was attached less firmly than by a peptide link. Protein-free solutions with the required properties can be obtained, but it is possible that they may have come from the impurities which are known to be present in crystalline pepsin. In any case the conclusive test of recombining the essential groups with the protein, as in the case of haemoglobin or flavoprotein, suffers in the case of pepsin from extreme technical difficulty and has not so far been accomplished.

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proteolytic enzymes which are unable to attack diketopiperazines can nevertheless hydrolyse cyclols, or that such enzymes can convert cyclols into ordinary peptides.

The frequency hypothesis of Bergmann and Niemann states that the number ( $a$ ) of amino-acid residues in a protein is expressed by the formula  $a = 2^m 3^n$ , where  $m$  and  $n$  are whole numbers. Now the average error in the determination of individual amino acids in a protein may be fairly taken to be 5-6%; assuming the possibility of an error of 6%, calculation indicates a very high degree of probability (approx. 80%) that a purely random distribution of amino acids should give values in apparent accordance with the formula of the frequency hypothesis. It appears, therefore, that so far as the complex globular proteins are concerned the hypothesis is not convincingly established, although in the case of the simple fibrous proteins it rests on a more secure basis.

#### S. J. PRZYŁECKI.\* *Protein symplexes.*

The molecules of many native proteins contain groups "apeptides" which are not composed of amino acids; thus serum albumin can be separated into fractions containing (a) exclusively amino acids (crystalbumin), (b) amino acids plus 10% carbohydrate (seroglycoid), (c) amino acids plus 4.8% carbohydrate plus 3.9% lipin; these three fractions have mol. wt. 70,000, and there is evidence of a fourth component with mol. wt. 100,000 containing amino acids plus 40% lipin plus 2% carbohydrate.

Such protein-apeptide complexes fall into the class designated symplexes by Willstätter and include substances of great physiological importance. The possible modes of combination between the protein and apeptide groups may be classified as follows.

(1) *Electrovalent symplexes.* These depend for their formation on the ionizable groups of protein and include such substances as nucleoproteins, compounds of basic and acidic proteins (e.g. protamine-insulin) and compounds of proteins with phosphatides and with acid and basic polysaccharide groups (bacterial antigens). Symplexes of this type are stable only within a certain pH range.

(2) *Covalent symplexes.* Compounds may be formed between proteins and carbohydrates (e.g. by N-glycoside linkages) or between proteins and fatty acids or lipins (e.g. by esterification); such covalent symplexes show a much greater degree of stability.

(3) *Co-ordination symplexes.* (a) *Polyosoproteins.* The protein-glycogen complexes isolable from animal tissues, similar substances from plants and

\* Communicated by letter, not read in person.



others which may be prepared artificially, exist by combination through the undissociated arginine and tyrosine groups of the protein; they are very labile but differ from the electrovalent symplexes in being stable at pH at which both components carry charges of like sign and in being stable in presence of salts.

(b) *Lipoproteins*. Similar symplexes, dissociable by organic acids, bases, or alcohols, according to their compositions but stable towards water and non-polar organic solvents, may be formed between proteins and fats, phosphatides, sterols or carotenoids, through the participation of the  $\text{NH}_2$ ,  $\text{COOH}$ ,  $\text{CH}=\text{CH}$ ,  $\text{OH}$  or  $\text{CONH}$  groups of the protein.

(c) *Polyproteins*. Labile associated aggregates may occur between the molecules of different proteins.

The above symplexes are to be regarded as chemical compounds in which the components combine in stoichiometric proportions; they seldom occur in nature as individuals, but are found as associations of several different types of symplexes; such a situation is illustrated by the case of serum albumin to which reference has already been made.

Apart from the above enumerated chemical compounds so-called pseudolipoproteins are known. These are due to purely cohesive forces between the apolar groups. They differ from the true, co-ordinational lipoproteids in being readily separated by apolar organic solvents such as benzene, light petroleum, etc.

Professor H. H. WEBER. *Muscle proteins and properties of the muscle.*

Muscle can easily be separated into four fractions of proteins:

(1) An albumin fraction—myogen—is soluble under all conditions in a much higher concentration than the muscle contains. Consequently it must exist in solution in the living muscle too. 14–15% of it can be mechanically squeezed out of the muscle. The whole amount of myogen extracted with 0.6 M KCl is 22% for the white muscle.

(2) A globulin fraction—globulin X—is soluble under physiological conditions in a far lower concentration than the living muscle contains. Therefore the greater part of it must form structures in the living muscle. In the press juice it amounts to only 2% of total muscle protein. In the KCl extraction it is also 22%. Both of these fractions (globulin X and myogen) are typical representatives of their classes of protein in nearly all properties (solubility, viscosity, mol. weight).

(3) An anomalous globulin—myosin—exists in the KCl extraction (ca. 40% of total muscle protein), but does not go into the press juice. It can easily be sharply separated from globulin X because the precipitation

of myosin is completely finished at 0.04 M KCl at *ca.* pH 7. The precipitation of globulin X begins at 0.005 M KCl at *ca.* pH 7. Solutions of myosin possess a high double refraction of flow, a high and anomalous viscosity, and they form a gel in concentrations *ca.* 2 %. All this shows us that myosin particles are rod-shaped molecular bundles optically anisotropic in themselves.

(4) Stroma proteins are insoluble even in concentrated salt solutions. Stroma is generally not optically anisotropic.

By certain methods myosin may be formed into threads. These are equal to the muscle in capability of swelling, tensile strength, elastic properties and X-ray diagram. They also possess double refraction of rods and are optically anisotropic in themselves. Their double refraction of rods quantitatively amounts to the quantity required by the theory of Wiener. In the muscle we observe only 40 % of both kinds of double refraction in comparison with the thread. On the other hand the muscle contains only 40 % myosin, and the volume of its doubly refracting disks is again about 40 %. Thus the Q-disks of muscle also consist of myosin rods oriented completely parallel with their long axis to the axis of the fibre. A dry myosin thread swelling in a myogen solution absorbs only water but no myogen. Therefore the spaces between myosin rods in normally swollen threads (20 % protein) must be  $< 70 \text{ \AA}$ , their number per  $\text{mm.}^2$  of the cross-section  $> 12\text{--}15 \times 10^9$ , the rod measuring  $< 45 \text{ \AA}$  in diameter and  $> 500 \text{ \AA}$  in length. According to Boehm's X-ray studies the distance between the filamentous molecules of myosin in the swollen thread is 11  $\text{\AA}$ . So the cross-section of one rod contains at most 17 molecules. The analysis of birefringence shows that completely elastic stretching of the oriented thread results from the stretching of its single rods. Filamentous molecules are evidently folded. From the transition of  $\alpha$  myosin into  $\beta$  myosin in the stretched state Astbury likewise concludes the folding of molecules. In general this is the knowledge we have at the moment of the structure of the Q-disk.

Dissolved myogen claiming only 20 % of muscle volume can be placed into the sarcoplasm where, for mechanical reasons, it is expected to be.

We cannot tell anything definite about the simply refracting disks and about the signification of globulin X and of stroma proteins.

From the fact that muscle fibrils are composed of filamentous molecules we may explain:

- (1) the capability of being highly stretched,
- (2) the changeable modulus of elasticity,
- (3) the thermoelastic effect,
- (4) the quantitative proportion between damped and undamped elasticity.

The contraction of muscle by heat is the same as that of the myosin thread between 40 and 70° with nearly the same contraction and tension, whereas active contraction of muscle results from a reaction with certain products of metabolism. Colloidal effects of products of metabolism are observed in a decrease of solubility of muscle protein in fatigued state. This effect is completely reversible. It cannot be explained by formation of lactic acid. It cannot be produced by adding creatine, or muscle adenylic acid to the minced muscle. This decrease results exclusively from a decrease in solubility of myosin.

Professor K. H. MEYER. *A contribution to the problem of protein denaturation.*

The insoluble solid silk thread arises, as is well known, from the water-soluble fluid content of the silk glands of the caterpillar; this coagulation occurs, as Foa (1912) has shown, as a result of stretching. We have examined this case of denaturation in greater detail; first, because the proteins involved are relatively simple and consist only of polypeptide chains, and secondly, because the structure of the crystallized thread is well known (the first three-dimensional model of a polypeptide chain (Meyer and Mark 1928) was based on a study of the X-ray diffraction pattern of silk).

M. Jeannerat and myself have made experiments on the silk glands and on aqueous solutions of the content of the glands; a detailed account of this work will appear in the *Helvetica chimica Acta*. As a result of drying, treatment with salt solutions, alcohol, acids, etc., the content of the gland becomes insoluble and shows crystal interferences. The freshly coagulated worm-like gland (ca. 8 cm. long) can be stretched reversibly seven to ten times its length—like a rubber band. If the gland (or its contents) is held in the stretched condition for 10–30 sec., it crystallizes and no longer contracts when released.

It is known that pure amorphous rubber is metastable at 0° C and takes months to crystallize if undisturbed. If stretched, however, the speed of crystallization is very much increased—as a result of the parallel orientation of the chains—so that the state of “supersaturation” is removed. Amorphous selenium behaves in the same way: it may be kept indefinitely in the undercooled condition, but becomes “elastic” like rubber at 72° C (Meyer and Sievers 1935) and crystallizes on stretching (Prins and Dekeyser 1937). After crystallization it is inelastic and is no longer soluble in sulphur.

From the analogous behaviour of these systems and that of silk we conclude that the silk gland contains a fluid in a metastable condition, that is, a supersaturated protein solution, the supersaturation of which is abolished by mechanical means.

Aqueous solutions of the content of the gland are also metastable; on standing they soon became covered by a solid skin of insoluble protein. In course of time they become more and more viscous and solidify spontaneously after several weeks. On stirring, shaking, or bubbling a gas through the solution, a fibrous protein is eliminated, leaving in solution another protein which soon gelatinizes. There are therefore at least two proteins present side by side in supersaturated solution in the content of the gland.

We may suppose that the known denaturation of surface films of other proteins discovered by Ramsden (1894) is caused in the same way: supersaturation is abolished as a result of the mechanical orientation of the chains under the influence of surface forces.

The observed elimination of "fibroin" from solution by whipping recalls the defibrination of blood. In the blood, fibrinogen gives rise to a supersaturated solution of fibrin, which is subsequently eliminated at surfaces. This elimination is accelerated by mechanical agitation (as in the case of silk fibroin).

It seems possible that other systems occurring in living organisms should be regarded as "supersaturated solutions", and that the properties of such may be of importance in biological processes.

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W. T. ASTBURY. *Evidence from the X-ray study of fibrous proteins.*

The natural protein fibres are polypeptide chain systems with the general direction of the chains running along the length of the fibre. The chains may be fully extended, as in fibroin and fibrin for example, or regularly folded, as in the hair protein keratin and the muscle protein myosin.

Keratin and myosin are similar in both molecular configuration and elastic properties.

With its  $\beta$  subgroup, the configurational group to which keratin and myosin belong comprises all the fibrous proteins of the epidermis and protective covering of animals, muscular tissue, and probably also myo-epithelial tissue. And the only other group of natural fibrous proteins revealed by X-rays is the collagen group, which includes connective tissue, tendons, elastin, fish skin and fins, etc.

The long-range stretching and contraction of the fibrous proteins points directly to the structure of the "globular" proteins. The various contracted states are but intermediate steps to more comprehensive states of folding.

When the configuration specific to a protein is disturbed, the protein is "denatured"; new non-specific configurations are formed which always include  $\beta$  chain-bundles. The transition from the specific to the denatured state must in general be very easy, and the X-ray indications are that the structure of the globular proteins is analogous to that of the folded fibrous proteins. The unfolding of myosin at comparatively low temperatures is a linear example of this.

It is possible from X-ray and density data to show that there is a stoichiometrical distribution of the amino-acid residues in the fibrous proteins similar to what has been found for the globular proteins, and it is also probable that the fibrous proteins, too, fall into the Svedberg scheme of multiple molecular weights. Again, just as there are in the Svedberg scheme apparently only a limited number of configurations independent of constitution, so there are considerable variations of constitution within both the keratin-myosin and collagen configurations. Constitution alone does not decide the configuration: there must be some factor common to the synthesis of all proteins, whether fibrous or globular.

Protein monolayers are formed also by the unfolding of specific configurations, leaving the side chains pointing normal to the substrate. In the form of built-up multilayers their thickness has been measured both by X-rays and by direct means -including the screw micrometer. The thickness of one monolayer of egg albumin is about  $9\frac{1}{2}$  Å, as would be inferred from X-ray data given by the natural fibrous proteins.

The spacing between successive nucleotides in thymo-nucleic acid is almost exactly equal to that between successive side chains in the  $\beta$ -proteins. Protamines like clupein thus combine directly with this acid to form optically negative fibres of the same period.

From combined X-ray and chemical data given by the fibrous proteins, the nucleic acids, and the tobacco mosaic virus, each nucleotide in the virus appears to co-ordinate about 54 amino-acid residues in a volume of about 8800 Å<sup>3</sup>.

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Dr G. BOEHM. The study of X-ray diagrams of (i) dried striated muscle, and dried bundles of stretched myosin fibres, (ii) of living skeletal muscle, and bundles of myosin fibres extended and soaked in phosphate buffer, shows that the X-ray diagram of muscle is entirely due to the myosin. This substance is present in muscle in the same state of orientation as in a myosin fibre which has been stretched "physiologically", i.e. without application of high temperature.

The equatorial interference points indicating a distance of about 11 Å disappear during isotonic contraction. From this one can conclude merely that the orientation of the myosin particles has been disturbed in some way. Since new interferences do not appear, no further conclusion can be drawn about changes in the myosin chains.

In good diagrams of living muscle, the equatorial interference points are just perceptibly differentiated and approach the central spot very closely. Attempts, however, to obtain several distinctly separate interferences failed, even with methods which had been successful in the case of X-ray diagrams of the tendon. It would appear, therefore, hardly possible at present to distinguish with this method between *inter*- and *intra*-micellar reactions in muscle, with a degree of certainty such as has been obtained, for example, with cellulose.

Such a distinction should be aimed at in the case not only of muscle, but of any structure which is partly composed of protein particles of unequal diameters. Such tissues, as is well known, do not contain separate chains of protein molecules, but grouped primary valency chains: micellae, which can be analysed easily especially if present within a regular structure. Probably, as the X-ray diagrams have shown in the case of cellulose, some agents act only upon the *inter*-micellar substance or at the surface of the micellae, others combine with each individual molecular chain, i.e. in the *intra*-micellar substance. This raises a special question in the case of muscular contraction. The "contraction substance" liberated presumably in the "intermicellar" space would have to act, according to current views, upon each individual primary valency chain of the myosin. This would introduce a finite time of "intra-micellar" diffusion which, at present, cannot be measured quantitatively.

Some indications can be found of "intra-micellar" reactions even in the not very distinct diagrams of the muscle, provided any change of shape of the myosin micellae is prevented. The diagrams of dried and living muscle show that some part of the water is present in the "intra-micellar" space. Furthermore, during rigor, and during isometric contraction of a tortoise muscle (8 min. exposure with Cu  $K_{\alpha}$ -radiation), the equatorial interference

points become a little more distinct which may be due to a "permutoidal" reaction of the micellae.

Further information on muscle, and other protein fibres, will presumably depend upon a combination of different, especially of optical, methods. If a clear distinction can be achieved between *intra* and *inter*-micellar reactions of such tissues, further light may be thrown also on a number of pharmacological actions.

Dr E. GORTER. Whoever studies the protein molecule by the spreading method must be well aware that he is dealing with molecules with a special shape, namely, that of flat platelets, having a thickness of 10 Å.

I shall first consider whether this special shape of the spread molecule is of a rare occurrence among proteins.

At first sight it would appear that a maximum spreading can only be obtained with a few samples of proteins, and even with those spreading occurs under special circumstances only, the most important of which are the electrolytic strength of the solution on which the protein is spread and the presence of bi- or tri-valent ions. In one group of proteins the spreading occurs easily in a short time at the isoelectric point and on very acid or alkaline solutions, even if they contain a small amount of electrolytes.

Other proteins show a greater tendency towards spreading in so far as the minima on each side of the isoelectric point tend to disappear.

They behave as if active polar groups  $\text{COO}^-$  and  $\text{NH}_3^+$  are covered by another substance. A complex protein prepared from ovalbumin and tartrazin shows this better spreading tendency at the acid side of the isoelectric point, because the  $\text{NH}_2$  groups have fixed a molecule of tartrazin. Among the good-spreading proteins of one of these two types we can mention the following: different albumins and globulins, insulin, pepsin, trypsin, globin. But there are also proteins which do not spread under the ordinary experimental conditions.

We found that beautifully spreading substances are obtained by adding a trace of a proteolytic enzyme to these. The split product behaves as a protein of the first or second group. As an example we can mention the spreading of fibrinogen and myosin. Perhaps the most interesting of this group is heat-denatured ovalbumin, which has no spreading tendency, but is again transformed into an easily spreading substance by the addition of a proteolytic enzyme. There exist also examples of proteins which do not spread because they contain too many soluble groups in the side chains. We never could get spreading of the purest clupein, sent us by Dr Linderstrøm-Lang. We may therefore conclude that most proteins can be induced to

spread and that the flat platelets shape of the molecule is not something exceptional.

Whoever studies proteins by the spreading method must also keep in mind that his experiments give him some information only of *one surface of the molecule*, in which the polar groups CO—, NH, COO— and NH<sub>3</sub> are lying, as this surface is in contact with the water in the tray.

On the other hand, we may be very satisfied that the spreading method enables us to study the one most important surface of the protein molecule with great precision.

Our conclusions that the method of spreading also enables us to collect important information about the properties of a protein molecule would be placed on a weak basis if we had to consider the protein molecules, when spread, as denatured.

Now one reason that this is not true is given by the behaviour of heat-denatured ovalbumin, which does not spread. Moreover, we were able to show that pepsin and trypsin remain active as a proteolytic enzyme after having been spread on a water surface.

Dr J. F. DANIELLI. Surface chemistry, in addition to giving us information about the structure of protein films, also gives a certain amount of information about the nature of the forces holding the polypeptide chain of the protein molecule in its specific configuration. Adsorption of protein molecules at an oil-water or an air-water interface may cause a reduction in free surface energy of the order of 35 ergs/sq. cm. The adsorption of a protein molecule is a practically irreversible process: it is almost entirely due to the hydrocarbon residues of the polypeptide chains. Similar forces involving a free energy change of the same order of magnitude will operate on the hydrocarbon residues of the protein in solution, tending to roll the polypeptide chain into a form where the hydrocarbon residues are in close contact and the polar groups are directed towards the aqueous phase. This process should be fairly complete, but the adhesions between the hydrocarbon residues in the case of such short chains will not, judging from other film experiments, be sufficient to maintain a solid, i.e. a specific, structure. The only other forces available are chemical linkages.

In a solution of a protein such as haemoglobin, individual protein molecules show little tendency to combine. But after spreading at a surface they tend to link up and form a two-dimensional network of polypeptide chains. The groups involved in this linking up are probably those which are broken when the polypeptide chain unrolls at the surface, i.e. they are probably the groups involved in the linkages responsible for maintaining the specific structure. If this is so we should probably be able



to obtain information about the nature of these groups by allowing adsorption of protein molecules to take place in the presence of a high concentration of reagents which specifically combine with certain types of groups. It thus seems probable that surface methods will enable us to investigate the nature of the bonds maintaining the specific structure of proteins.

DOROTHY CROWFOOT. *X-ray studies of protein crystals.*

The number of protein crystals studied by X-ray methods is still small, chiefly owing to the difficulties of applying this technique to crystals of such low X-ray reflecting power. Most of the crystals also readily lose water on exposure to air, forming new collapsed crystal structures in which the arrangement of the units, as indicated by further decrease in the intensity of the X-ray reflexions, is considerably disorganized. So far, of only seven proteins—pepsin, insulin, excelsin, lactoglobulin, haemoglobin, chymotrypsin, and tobacco seed globulin—have sufficient X-ray measurements been made to cover even the first stages of crystallographic examination, the determination of unit cell size and cell molecular weight. And of these only three, lactoglobulin, haemoglobin, and chymotrypsin, have been studied both wet and dry.

These measurements bear in two directions on the existence of proteins as large molecules within the crystals. In the first place it is possible to calculate the maximum molecular weights of the protein units present, and these agree well with the molecular weights measured by the ultracentrifuge method. Secondly, the cell dimensions of the different protein crystals, particularly when dry, show certain regularities among themselves. They are commonly multiples of about 30, 50, and 60–70 Å, the crystals falling into two main groups in which the packing of the units is pseudo-cubic and pseudo-hexagonal respectively.

Whether these units should be considered as chemical molecules cannot be decided at this stage from the X-ray data. It is, however, possible to derive some information on their internal structure through a study of the intensities of the X-ray reflexions. Series of Patterson-Harker Fourier syntheses may be formed, using the observed intensities, and plotted as diagrams in which the peaks show the vectorial distribution of interatomic distances within the unit cell. This has been done for dry insulin and, in a preliminary way, for wet and dry lactoglobulin. For dry lactoglobulin the plot is very simple and shows only the main distribution of molecular centres; while those for wet lactoglobulin are complicated by the possible superposition of inter- and intra-molecular distances. The insulin patterns

are best suited for further analysis since the peak positions here must crystallographically be due only to structure within the Svedberg unit. But, as dry crystals only have been measured, the X-ray data are still very imperfect, and these patterns cannot be expected to give information on the actual placing of individual atoms. They evidently correspond to a fairly large-scale structure within the unit and suggest some kind of rhombohedral packing of subunits at the characteristic distances of 10 or 20 Å apart. Such a distribution seems significant in view of the measurements made by other methods on protein fibres and films.

Professor J. D. BERNAL, F.R.S. *X-ray evidence for the structure of the protein molecule.*

A logical method of attack on the problem of the protein molecule by X-ray analysis begins with a study of the available observations and deduces from them the range of possible structures which can account for these observations. So far unique solutions are not to be expected and cannot indeed be found, but the choice of structures can be still further narrowed by invoking our knowledge of the chemical and physico-chemical properties of proteins and of the lengths of bonds and of atomic radii established by X-ray analysis of simpler structures.

The existence in protein crystals of molecules of the size found in solutions by centrifugal methods can be considered as probable, though not proved. The contents of a repeat unit of all protein crystals hitherto examined has always been found to be a simple multiple of a Svedberg unit. The crystal unit can therefore not be larger than the unit in solution but it may well be a submultiple of it. Indeed, in the case of haemoglobin it appears that the crystal unit is half the molecule found in solutions. The possibility that protein crystals are made of units of smaller size and that a determinate number of these aggregate on passing into solution must be seriously taken into account. The remarkable swelling properties of protein crystals can be accounted for either on this supposition or on the hypothesis that the larger protein molecules are not rigid, but can themselves swell and shrink reversibly.

The chief features in the X-ray scattering of both crystalline, fibrous, and denatured proteins are the strong reflexions in the neighbourhood of 10 and 4.5 Å. These have been shown by Astbury in the latter cases to correspond to the side-chain and "backbone" distances between peptide chains. An analogous explanation must hold in the crystalline proteins, since experiments by Astbury, Dickinson, and Perutz have shown that actual atomic movements in denaturation of protein crystals are very

limited. Detailed analysis of intramolecular structure are only possible in the case of one protein—insulin—as here alone there is only one Svedberg unit in the cell. Unfortunately, only the dry crystal has been studied, and thus not only has all fine structure been lost but a distorted picture is given of the



FIG. 1a. Patterson projection of insulin structure on basal plane (after D. Crowfoot).

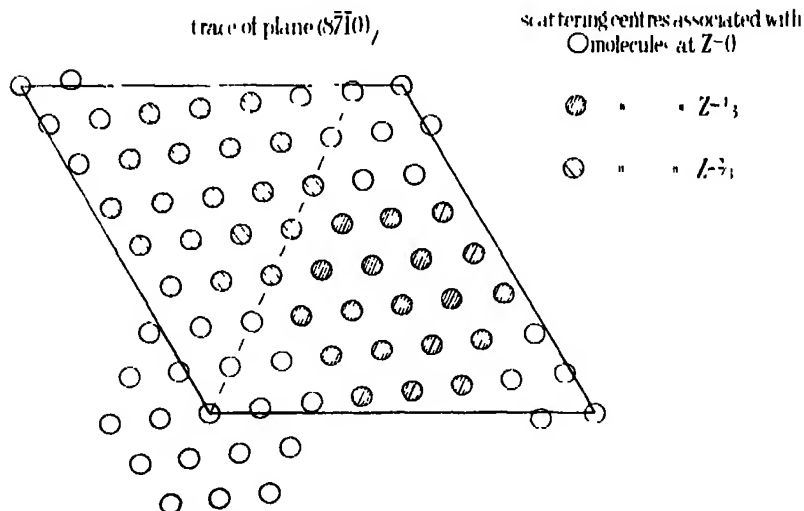


FIG. 1b. Idealized projection of insulin structure on basal plane showing positions of hypothetical scattering centres.

coarser structure. This can be partially corrected by introducing a disorder factor, and the resulting Patterson projections (see fig. 1a) give us a basis for a rough analysis. The whole pattern may be reduced to six significant peaks (three in the basal projection). This indicates that we may be dealing with concentrations of scattering matter approximately 10 Å apart. To find their positions we must depend not on *a priori* structures with arbitrary assumptions, but on an exhaustive study of the number of point combinations that

can give the observed peaks. It is immediately apparent that the peak vectors correspond to those of a body-centred cubic array. In projection on the basal plane this gives a hexagonal pattern. The whole of the lattice space can be filled by sets of hexagons containing two layers of points (see figure). It is a remarkable fact that for closed packing the plane joining nearest points is precisely the  $(8\bar{7}\bar{1}0)$  plane which is one of the strongest in the crystal. Each of the points in the projection may be occupied by 0, 1, or 2 equivalent scattering centres at different heights. These can be distributed in a large number of different ways, but only a few of these with 12, 18, or 24 centres will give the order of intensity of the peaks. Between these it is impossible to choose on X-ray grounds alone. The most we can say therefore at present is that plausible structures can be postulated for the insulin molecule in which it is constituted, most probably, of 24 submolecules.

We have now considerable confirmative evidence of a physico-chemical nature for this assumption, as protein molecules have been split into submolecules by various agents. As to their structure, we can legitimately speculate without the addition of any new assumptions that they consist of regularly folded peptide rings containing possibly twelve amino-acid residues held in shape by hydrogen links between adjacent CO and NH groups. Models of these can be made which have external dimensions of about 10 Å and prominent internal distances of about 4 Å. A protein molecule built up in this way would not only account for the X-ray evidence but would give a plausible explanation of denaturation phenomena. Reversible denaturation would consist of the separation and possibly the unfolding of the submolecules; irreversible denaturation in their conversion into long-chain molecules by the familiar mechanism of ring-chain polymerization.

Much research, however, will be required before any model can be put forward as anything more than a plausible working hypothesis; in particular, the structure of wet insulin crystals needs to be studied. Far greater co-ordination is needed between research by X-rays and by other methods. It would be of enormous value to have some form of central bureau for protein research which would facilitate exchange of information and material in this field, and assist in an ordered attack on the whole problem.

*[Note added in proof, 30 January 1939. It now seems probable, as the result of the work of Langmuir and recent observations on tobacco mosaic virus, that the forces between molecules in some protein crystals are due to the ionic atmosphere surrounding them in solution.]*

Recent discussion has shown that the interpretation of protein Patter-

son peaks by concentrations of scattering matter inside the molecule provides only one out of an infinite number of solutions. As it is still impossible to find, notwithstanding some claims that have been made, a satisfactory point solution both of the projections and the Patterson-Harker sections, it would seem probable that some more complex solution will have to be attempted.]

**J. MARRACK.** *Immunological aspects.*

Evidence that has accumulated in recent years shows that antibodies are modified serum globulins; the majority of the more effective antigens are proteins. The problems of immunochemistry are therefore intimately bound up with those of the structure of proteins.

Our knowledge of the relation between specificity in immunological reactions and chemical structure has been derived, mainly, from studies with synthetic antigens. The majority of synthetic antigens used are formed by attaching a relatively small group to a protein. The antibodies produced react specifically with these small attached groups (determinant groups). This suggests that the determinant groups of natural protein antigens might be similar small groups (prosthetic groups). Also the "combining groups" of antibodies might be similar prosthetic groups.

If such prosthetic groups were present in protein and were polar, they should project into the water when the protein was spread as a solid film at water-air or water-oil interface. The "combining groups" of the antibody of type II antipneumococcal serum, that combines with type II polysaccharide, should be polar, as they combine with the polar glucuronic acid of the polysaccharide.

In experiments by Dr Danielli, Mrs Danielli and myself (1938) purified type II antibody, over 90 % of which was precipitable by polysaccharide, was spread on a water-air or water-oil interface forming an elastic solid film. The surface area and surface potential of this film were not affected by the introduction of type II polysaccharide into the bulk of the water phase. There was therefore no evidence of combination between the polysaccharide and the antibody in the film.

There was also no evidence of combination between horse-serum globulin similarly spread as a film and an antibody to this protein, when the latter was introduced into the bulk of the fluid.

It is inferred that the combining groups of the antibody and determinant groups of the protein antigen are *not* small prosthetic groups, but specific patterns of certain amino acids, which are disarranged when the proteins are unrolled as a film. This fits the experiments of Landsteiner and van der Scheer (1932, 1934) and the older experiments of Dakin and Dudley (1913).

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Dr E. HOLIDAY. *Breakdown products of antigen.*

In quantitative immunological experiments it is an ideal requirement that the antigen should be antigenically homogeneous. Proteins obtained by electrophoresis and shown to be homogeneous in the ultracentrifuge come as near to this requirement as has so far been possible.

Having immunized an animal (e.g. rabbit) with such a purified protein it is then of interest to determine how far one can degrade the antigen (e.g. by enzymic digestion) before affinity for antibody is lost.

In an experiment a highly purified preparation of horse-serum albumin was subjected to digestion by 1/4000 parts by weight of pepsin at pH 2.1 for periods of 5 and 30 min. The digests were neutralized and then subjected to the following procedures, the results of which are drawn up in tabular form.

	Albumin	5 min. digest	30 min. digest
Ultracentrifuge	Homogeneous $s = 4.2 \times 10^{-1}$	Homogeneous $s = 1.95 \times 10^{-1}$ . Probably exists as $\frac{1}{2}$ molecules	Homogeneous $s = 1.14 \times 10^{-1}$ . Probably exists as $\frac{1}{3}$ molecules
Electrophoresis	One component. Mobility at pH 8.0: $-7.6 \times 10^{-5}$	Two components Mobility at pH 8.0: (1) $0.3 \times 10^{-5}$ (2) $-9.0 \times 10^{-5}$	Two components. Mobilities not measured
Dialysis	Undialysable	< 10% of nitrogen dialysable. Dialysate contained over 90% of the tryptophano of the albumin	—
Precipitin reaction	Precipitate + + + +	Precipitate + + +	No precipitate. Partially inhibited precipitation of unaltered albumin

The experiment seems to indicate that pepsin is a useful enzyme for degrading proteins for this type of experiment. It splits successively by dividing into (?) halves, quarters and eighths. In these early stages small fragments are split off which include most of the tryptophano in the molecule of serum albumin. At the stage of division into eighth parts there is still evidence of affinity between the degradation products and antibody.

## Carbohydrate metabolism and muscular exercise

BY F. C. COURTICE, *Beit Memorial Research Fellow.*

C. G. DOUGLAS, F.R.S., AND J. G. PRIESTLEY

*From the Physiological Laboratory, Oxford*

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Courtice and Douglas (1936) showed that, although a prolonged period of moderate muscular work may be associated with some rise of respiratory quotient and an enhanced carbohydrate oxidation, a persistent low respiratory quotient and ketonuria may result during subsequent rest. They concluded that this low quotient and ketonuria were in the main attributable to the reduced ratio of carbohydrate to fat oxidized owing to the depletion of readily available carbohydrate in the body as a result of the preceding muscular exercise. A second period of exercise was, however, still capable of causing a distinct rise of the respiratory quotient which was maintained throughout the exercise, to fall again abruptly to a persistent low level when the exercise stopped. Finding in one subject (Courtice) that after the ingestion of sucrose, glucose or fructose during post-exercise rest there was distinct evidence of a reduction of sugar tolerance in comparison with observations made during an initial resting period before exercise, they suggested the possibility that the activity of the endocrine organs associated with carbohydrate metabolism may be correlated with the varying activity of the muscles and so afford a partial explanation of the changes of carbohydrate metabolism which result from muscular exercise.

Mills (1938) confirmed on Douglas the reduction of glucose tolerance which ensues after a prolonged period of muscular exercise, but in his own case obtained inconstant results, some experiments showing a lowered glucose tolerance, others no significant change. He also examined the influence of the carbohydrate content of the diet taken previous to the experiments, and tested the influence of intravenous injection of insulin on the subject without any previous exercise and after exercise.

We have now been able to continue these experiments in greater detail, and in particular to determine the respiratory exchange and quotient continuously by the bag method whilst obtaining blood samples for the determination of blood sugar, lactic acid and  $\text{CO}_2$  combining power.

The exercise was the same as in the original experiments of Courtice and Douglas, viz. a walk of 10 miles on practically level ground at the rate of  $4\frac{1}{2}$  m.p.h. In a few experiments the distance was increased to 15 miles. In every experiment the subject was in the post-absorptive state at the start. Courtice and Douglas acted as subjects.

## METHODS

*Glucose tolerance.* Preliminary determinations of the respiratory exchange and blood sugar were first made. Immediately afterwards 50 g. of glucose dissolved in 300 c.c. of water were taken and the respiratory exchange was determined continuously (by a succession of bags) during the first hour, and subsequently during the last 10 min. of each quarter of an hour for a further hour and a half. All samples of expired air were collected for approximately 10 min., and in the figures the respiratory quotient is plotted at the mid-point of each of these periods. The blood sugar was determined at 5 min. intervals for the first half hour, and subsequently at 15 min. intervals for a further two hours.

*Reaction to insulin.* Insulin solution (Burroughs, Wellcome and Co., made with crystalline insulin) was injected intravenously into the forearm, the respiratory exchange being determined continuously for one hour, twelve 5 min. bag samples being taken, and the blood sugar at 4 min. intervals for one hour.

*Reaction to adrenaline.* Adrenaline chloride (Parke, Davis and Co., 1:1000 solution) was injected subcutaneously into the upper arm. The respiratory exchange was then determined by a succession of 10 min. bag samples for  $2\frac{1}{2}$  hr., with the exception of a 10 min. interval after the end of each half-hour. Blood-sugar determinations were made at 10 min. intervals for the first hour and subsequently every quarter of an hour.

In all experiments the determinations were made with the subject reclining at rest in a deck-chair. In experiments after muscular exercise the preliminary observations were made after the subject had been resting for 1 hr., so that the glucose or insulin was actually administered about  $1\frac{1}{2}$  hr. after the exercise stopped.

Blood sugar was estimated in samples of capillary blood taken from the finger by puncture, after the hand had been warmed in water at about  $45^{\circ}\text{C}$ , by the method of Hagedorn and Jensen as given by Peters and Van Slyke (1932), except for the use of Whatman no. 44 filter papers in place of cotton-wool for filtration.



Blood lactic acid was determined in duplicate analyses by the method described by Friedemann, Cotonio and Shaffer (1927) and Friedemann and Kendall (1929). Blood was taken for this purpose from a vein in the forearm, coagulation and glycolysis being prevented by the addition of 0.2 % potassium oxalate and 0.1 % sodium fluoride. The blood was kept on ice from the time of withdrawal until required for use.

The  $\text{CO}_2$  combining power of the same blood was determined by the blood-gas apparatus designed by Haldane (1920) which was completely immersed in a water bath, the blood being first equilibrated at  $37^\circ \text{C}$  with air containing  $\text{CO}_2$  at a partial pressure of approximately 40 mm. Hg.

It should be noted that the curves given in the figures frequently represent the average results of two or more experiments. As the samples for analysis were always taken at the same time intervals in such cases, and the individual curves resembled one another very closely, such a method of representing the results can be safely used.

#### THE EFFECT OF EXERCISE ON GLUCOSE TOLERANCE

We have confirmed the reduction of sugar tolerance observed in the original experiments of Courtice and Douglas as well as in those of Mills. Fig. 1 shows the effect on the blood-sugar concentration and respiratory quotient after the ingestion of 50 g. of glucose without preliminary exercise and after prolonged moderate exercise. Both subjects reacted similarly.

The rate at which the blood-sugar concentration rises is practically the same, no matter whether or not exercise has preceded the test, but after exercise the curve rises for a longer period and reaches a higher level, so that the area enclosed is greater than in experiments without preliminary exercise. Once the blood-sugar concentration begins to fall, it falls rapidly and in both cases shows a negative phase between the 100th and 150th minutes. This figure shows the average results of three experiments on Courtice and two on Douglas both without and after exercise, but in individual instances blood-sugar concentrations as low as 65 mg /100 c.c. have been found during this negative phase. We occasionally had a slight glycosuria in experiments after exercise. Mills found that in some of his experiments the blood-sugar concentration rose more slowly after exercise than when there was no preceding exercise, and attributed this to delayed rate of absorption from the gut, but in our present series of experiments there is little or no evidence of this.

There is a definite delay while the blood-sugar concentration is increasing after the ingestion of glucose before the respiratory quotient begins to rise.

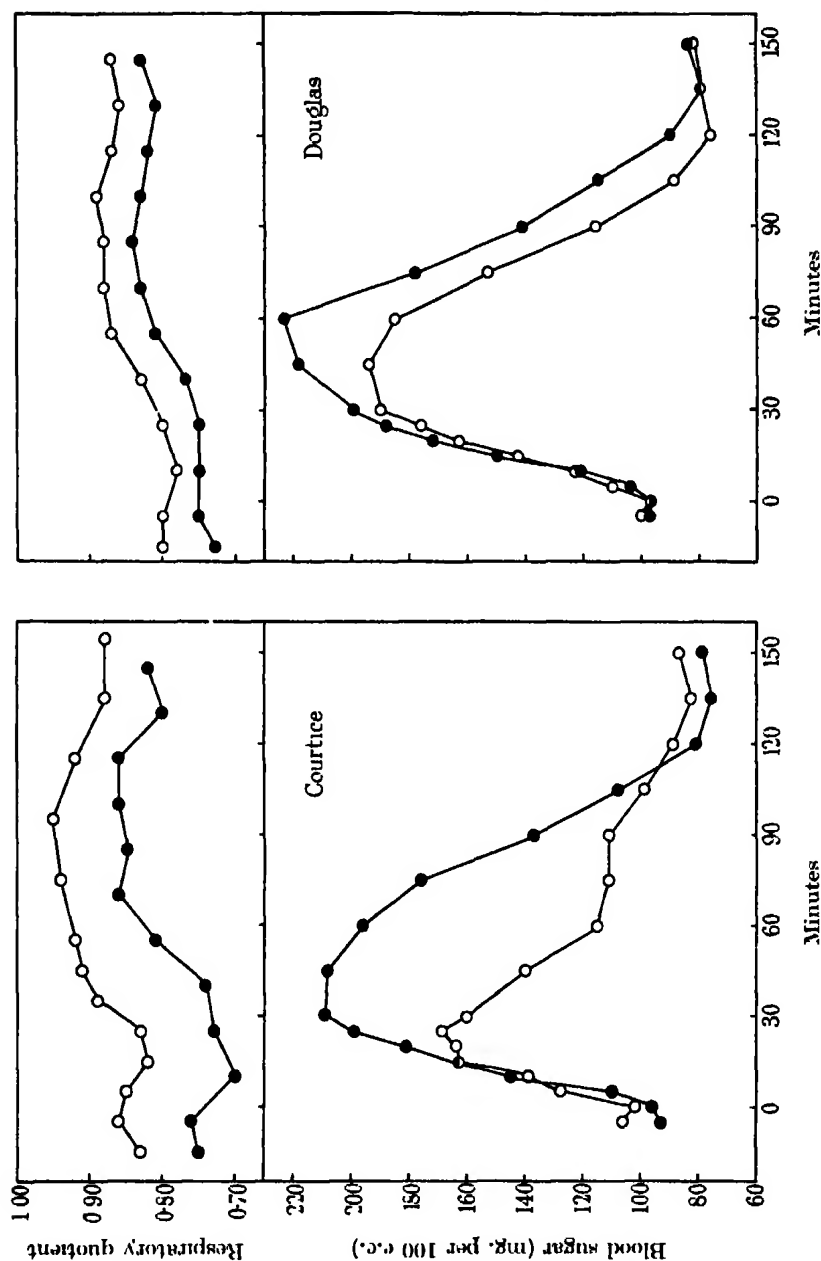


FIG. 1. Effect of ingestion of 50 g. of glucose on the blood-sugar concentration and respiratory quotient. O without preceding exercise; ● after exercise. Courtice: average of three experiments in each case. Douglas: average of two experiments in each case.

Indeed, as will be seen in fig. 1, there may be a fall of quotient at this time. Attention has been drawn to this fall by Tögel, Brezina and Durig (1913), and it is also apparent in a figure given by Higgins (1916) and is suggested in some of the experiments of Cathcart and Markowitz (1927). Tögel, Brezina and Durig state that the fall of quotient is due to a rise in the oxygen consumption without alteration of  $\text{CO}_2$  output and is not caused by mere modification of the breathing, and our own results are in general agreement with this. A clear rise of the respiratory quotient above its preliminary resting value does not become evident until the blood-sugar concentration has attained its maximum. This is more clearly shown in fig. 2, and the matter will be discussed in relation to this figure. The rise of respiratory quotient and its subsequent fall follow the same course in the experiments with and without preceding exercise if allowance is made for the lower initial level in the latter case, and are accompanied by the usual increase and decrease of the total respiratory exchange which result from the ingestion of sugar.

It is well known that the glucose tolerance is affected by the amount of carbohydrate in the preceding diet, being increased by a diet rich in carbohydrate and poor in fat, and decreased by a diet poor in carbohydrate and rich in fat. As Courtice and Douglas have brought forward evidence that the persistent low respiratory quotient after prolonged exercise is largely determined by a reduction in the proportion of carbohydrate to fat oxidized in the body, we decided to test the effect of changes of diet in our own case to see how a change of tolerance brought about in this way would compare with the change resulting from prolonged exercise. Mills has shown in Douglas's case that if a diet rich in carbohydrate is taken the sugar tolerance is increased both in experiments made without exercise and after exercise. We have now tested in Courtice's case the effect on the glucose tolerance of a high-carbohydrate, low-fat diet and of a low-carbohydrate, high-fat diet. In the case of the high-carbohydrate diet the amount of fat taken was cut down so far as possible and the subject lived mainly on foods rich in carbohydrate such as bread, potatoes, jam and sucrose for 6 days, with the result that the initial post-absorptive respiratory quotient was 0.94-0.96. A diet closely approximating to the diet no. 7 of Himsworth (1935) was chosen for the low-carbohydrate, high-fat diet, giving a daily intake of 55 g. of carbohydrate, 94 g. of protein and 220 g. of fat, with a total energy value of about 2576 kcal. This diet caused a marked and continuous ketonuria and an initial post-absorptive respiratory quotient of 0.74-0.75: it was maintained for 9 days. In fig. 2 the influence of these diets on the glucose tolerance is contrasted with the results obtained both without

exercise and after a 15-mile walk at  $4\frac{1}{2}$  m.p.h. when the subject was taking his normal diet.

In this instance the high-carbohydrate diet makes little or no difference to the glucose tolerance, although during the test the respiratory quotient remains at a much higher level throughout than in tests made when the subject was taking his normal diet. On the other hand the decrease of sugar tolerance after 9 days of the low-carbohydrate, high-fat diet is

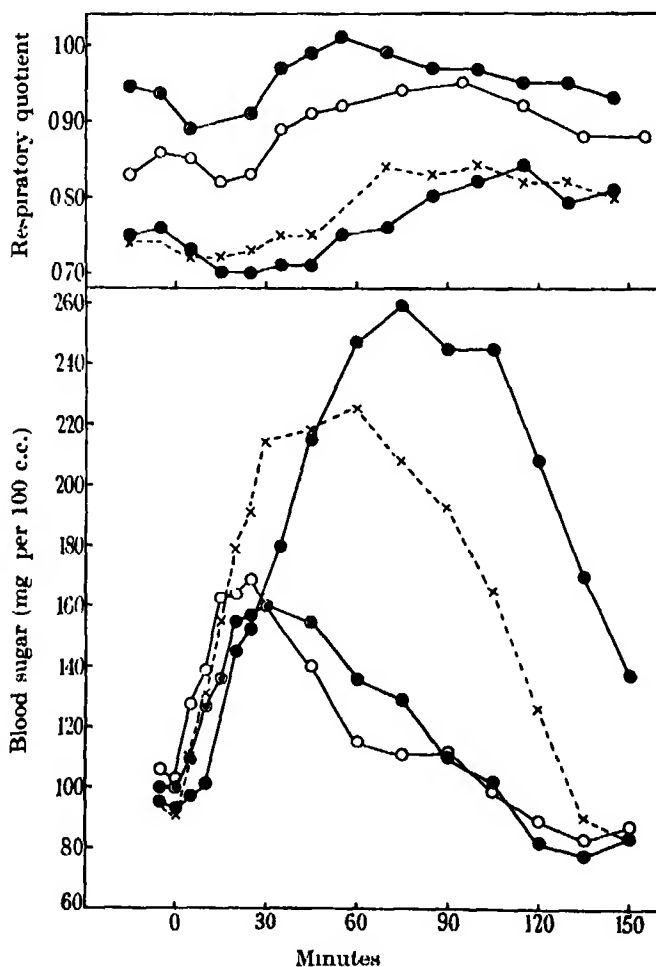


FIG. 2. Subject Courtice. Effect of ingestion of 50 g. of glucose on the blood-sugar concentration and respiratory quotient. ○ Normal diet, without exercise (average of three experiments); ◐ high carbohydrate diet, without exercise (average of two experiments); ● low-carbohydrate, high-fat diet, without exercise; × normal diet, after 15-mile walk.

striking. The sugar tolerance curve after the 15-mile walk bears a close general resemblance to the tolerance curve on this diet, though the actual decrease of tolerance is not quite so great. There was marked ketonuria after the exercise.

Fig. 2 shows, even more clearly than fig. 1, the delay which occurs after the ingestion of glucose before there is any sensible increase in the respiratory quotient. This delay, which is about  $\frac{1}{2}$  hr. in the experiments without exercise with normal and high carbohydrate diets, is of the order of 1 hr. in the experiments with a low-carbohydrate, high-fat diet and after exercise with a normal diet. While the blood-sugar concentration is rising the respiratory quotient actually falls, and it only begins to rise as the blood-sugar concentration passes its maximum, and attains its highest value after the blood-sugar concentration has begun to fall. It looks therefore as though the rise of blood-sugar concentration *per se* does not cause the rise of respiratory quotient, but this depends on the effective development of the processes for the disposal of excess sugar which shows itself in the rapid fall and final negative phase of the blood-sugar concentration.

Whether these processes, including, no doubt, increased insulin secretion, are gradually developing whilst the blood-sugar concentration is rising, or only begin when the blood-sugar concentration has risen to some definite level (an argument which might be supported by the linear rise of sugar concentration), we do not know. But in this connexion it is interesting to note that whilst the glucose tolerance is reduced, and sometimes greatly reduced, when determinations are made an hour and a half after the cessation of exercise, the tolerance may be but little altered from normal if glucose is ingested *immediately* after stopping the exercise, as is shown in fig. 3. Nevertheless, in this case, although urine secreted during the exercise never gave a positive Rothera reaction, urine passed at the end of 30 min. after taking the glucose always showed a strongly positive Rothera reaction, the ketonuria disappearing gradually during the next hour. As the ketonuria developed whilst the blood-sugar concentration was rising to its maximum this suggests that there can be no great increase in the oxidation of carbohydrate in the body during this period, which is thus in correspondence with the behaviour of the respiratory quotient.

#### THE REACTION TO INSULIN AND ADRENALINE

Mills has tested on Douglas the effect of the intravenous injection of 3 units of insulin both without preceding exercise and after exercise, and

found no appreciable difference in the response save that in the latter case the return of the blood sugar to normal seemed to be a little delayed. In these experiments the blood sugar fell in about 20 min. to 45 mg./100 c.c. while a dose of 5 units scarcely caused any greater fall. We therefore decided in the present series of experiments to reduce the dose of insulin to 2 units,

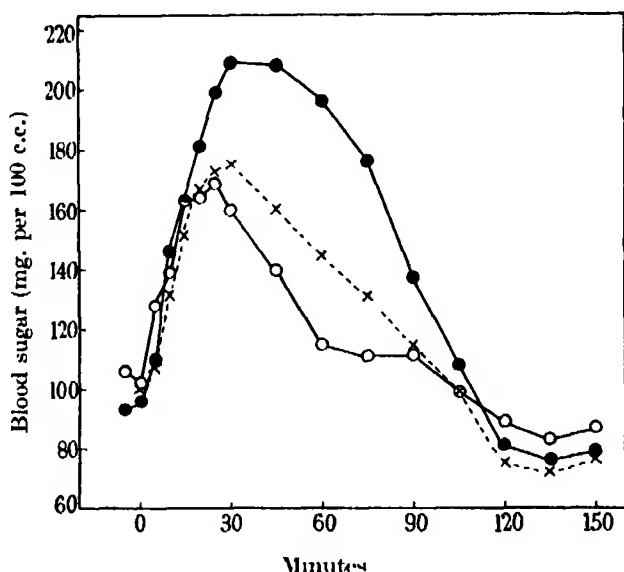


FIG. 3. Subject Courtice. Effect of ingestion of 50 g. of glucose on the blood-sugar concentration. ○ Without preceding exercise; × immediately after exercise; ● 1½ hr. after exercise. Each curve shows the average of three experiments.

so that the full effect could be followed without the risk that the blood sugar would reach a level below which it could not be depressed. This dose never caused any appreciable symptoms in Douglas when he was reclining at rest, but Courtice always showed characteristic symptoms of perspiration, slight faintness, and a trifling disturbance of vision. As he appeared to be more sensitive than Douglas to insulin (he is 28 years younger) we reduced the dose to one unit in his case, and with this dose symptoms were barely appreciable while he was at rest.

Fig. 4 shows the results obtained on the two subjects, in the one case without any preceding exercise and in the other an hour and a half after exercise. After the injection of insulin the blood sugar remains constant for about 4 min. and then falls to its lowest level, of 50–55 mg./100 c.c. in Courtice and 55–60 mg./100 c.c. in Douglas, in about 25 min., to rise rapidly in the next 15–20 min. and then remain moderately steady up to the

60th minute at a level slightly below the initial value. On the whole the behaviour of the blood sugar after insulin is practically identical, no matter whether the experiment is made without preliminary exercise or an hour and a half after exercise, although there is a little delay in the return to normal in the latter case with Courtice.

In control experiments in which no insulin was injected, but samples for blood-sugar determinations were taken at the usual intervals, the

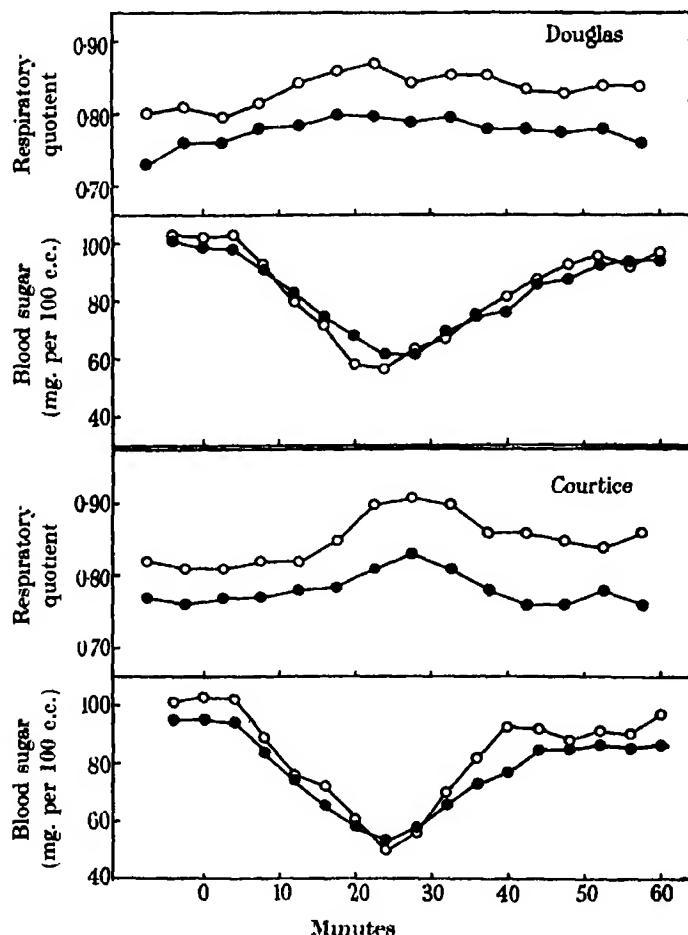


FIG. 4. Effect of the intravenous injection of insulin on the blood-sugar concentration and respiratory quotient. Dose of insulin, 2 units for Douglas, 1 unit for Courtice. ○ Without preceding exercise, ● after exercise. Each curve shows the average of two experiments.

respiratory exchange hardly differed appreciably from that found in normal post-absorptive observations on the same subject, except that sometimes there was a slight temporary increase immediately after puncturing a vein to represent the insulin injection. The respiratory quotient, too, remained unaltered.

After insulin injection Courtice's oxygen consumption in some cases showed a distinct, though inconsiderable, increase lasting for about 40 min., but in other cases no material alteration. Douglas showed much the same

TABLE I. AVERAGE OXYGEN CONSUMPTION C.C./MIN.  
IN CONSECUTIVE PERIODS OF 10 MIN.

		Insulin experiments					Control
		Courtice					
Before insulin	226	222	205	226	220	—	
	224	223	202	228	220	206	
After insulin	269	265	225	222	224	226	
	269	250	218	237	235	224	
	260	253	213	226	230	231	
	241	238	220	228	239	226	
	218	239	221	229	237	226	
	217	240	218	230	228	234	
		Douglas					
Before insulin	234	234	238	237	238	227	
	228	233	228	242	239	230	
After insulin	245	250	246	243	249	245	
	239	247	240	240	247	236	
	229	244	237	234	247	225	
	233	240	245	227	249	227	
	244	233	250	234	245	229	
	237	231	249	236	248	225	

reaction. The results of one control and five insulin experiments on each subject are shown in Table I. Notwithstanding the slight or negligible alteration in oxygen consumption the respiratory quotient always showed a clear increase lasting for some time, an increase that was more evident in Courtice than in Douglas. This increase was a little greater in experiments without preliminary exercise than in those after exercise: in other respects the behaviour of the quotient is the same in the two cases. The quotient begins to rise about 15 min. after the injection of insulin in Courtice's case and reaches its maximum about the time that the blood sugar is at its lowest: in Douglas's case it begins about the 8th minute and is still evident at the 60th minute.



Himsworth (1933, 1935), in his experiments on the influence of different diets on the reaction to insulin, has suggested that on a low-carbohydrate, high-fat diet there is evidence of reduced sensitivity to insulin since after the intravenous injection of insulin there is a longer delay before the blood sugar begins to fall, and the fall is slower and does not reach so low a level as in the case when the subject is living on a high-carbohydrate diet.

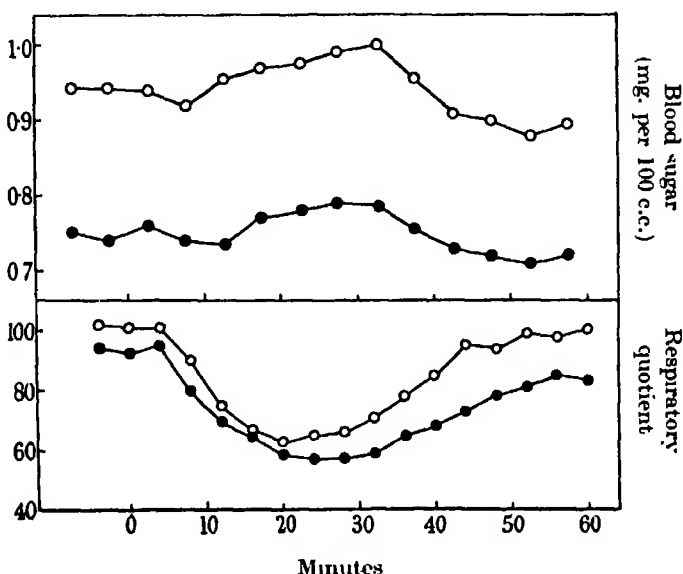


FIG. 5. Subject Courtice. Effect of 1 unit of insulin intravenously on the blood-sugar concentration and respiratory quotient. No preceding exercise. ○ Carbohydrate diet; ● low-carbohydrate, high-fat diet.

As our experiments, as well as those of Mills, on the effect of insulin after prolonged exercise, gave no evidence of such decreased sensitivity, we have tested on Courtice the influence of alteration of his diet. Fig. 5 shows the effect of insulin injection in one case when he had been living on a high-carbohydrate diet for 6 days, and in the other case when he had been living on a low-carbohydrate, high-fat diet, as described previously, for 9 days. It will be seen that in each case the blood-sugar concentration remains steady for the first 4 min. and then falls at a similar rate to about the same degree, making allowance for the slightly different initial values in the two cases, although the subsequent rise of blood-sugar concentration is slower in the case of the low-carbohydrate, high-fat diet. There is therefore in this instance no evidence of decreased sensitivity

such as Himsworth found. The respiratory quotient again shows a temporary rise as a result of the insulin injection, although of course the quotient in experiments on a low-carbohydrate, high-fat diet is throughout at a much lower level than on a high-carbohydrate diet.

The rise of respiratory quotient that occurs after the injection of insulin seems to us to imply an increased metabolism of carbohydrate, no matter whether it is an oxidation of carbohydrate or conversion of carbohydrate to fat. At all events it is not due to the formation of excess lactic acid with a resultant hyperpnoea, for in fig. 6 it will be seen that both the lactic acid content and  $\text{CO}_2$  combining power of the blood remain practically unchanged while the blood-sugar concentration falls and the respiratory

TABLE II

	Alveolar $\text{CO}_2$ pressure mm. Hg
Before insulin	42.5 41.2
Minutes after insulin:	
10	42.1
20	41.3
30	41.0
40	42.3
50	41.7
60	40.8

quotient rises. Nor have we been able to find any change in the alveolar  $\text{CO}_2$  pressure suggesting that the change in the respiratory quotient may be due to a temporary disturbance of the breathing resulting from such a cause as the uneasiness due to slight symptoms of hypoglycaemia. Mills tested this on Douglas, and Table II shows the average results obtained with Courtice in two experiments, the alveolar samples being taken at intervals of 10 min. for the hour succeeding the injection intravenously of one unit of insulin. In both these experiments he experienced distinct, though slight, symptoms of hypoglycaemia between the 24th and 40th minutes, but his alveolar  $\text{CO}_2$  pressure remains unchanged.

Insulin is but one of the endocrine secretions concerned with carbohydrate metabolism, and for comparison we have tested on ourselves the effects of adrenaline given subcutaneously, and of simultaneous subcutaneous injection of adrenaline and intravenous injection of insulin. The general effects of adrenaline in this connexion, e.g. rise of blood

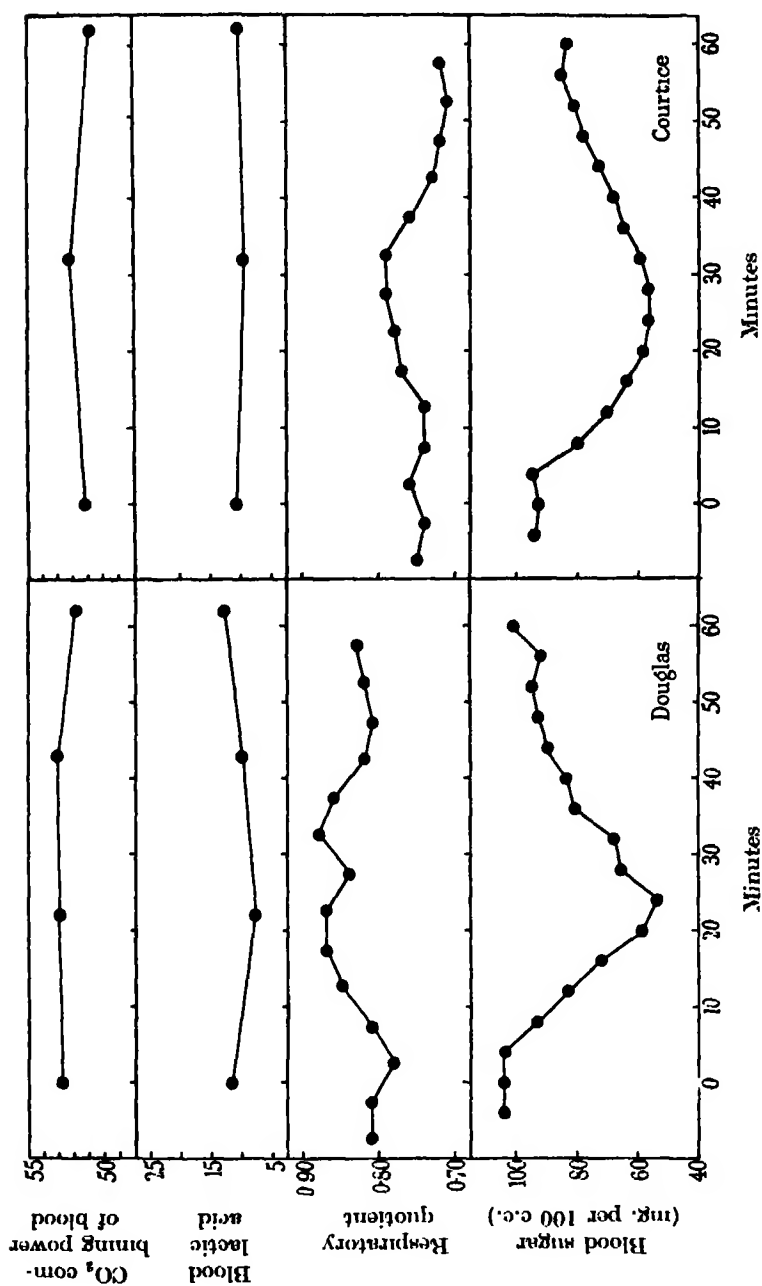


Fig. 6. Effect of the intravenous injection of insulin on the blood-sugar concentration, respiratory quotient, blood lactic acid (in mg./100 c.c.), and CO<sub>2</sub> combining power of the blood at 40 mm. CO<sub>2</sub> pressure (in c.c. CO<sub>2</sub>/100 c.c.). Dose of insulin, 2 units for Douglas, 1 unit for Courtoise.

sugar and formation of excess lactic acid are of course well known (cp. for instance, Cori, 1931).

The results of our experiments are shown in figs. 7 and 8. After the injection of 0.5 mg. adrenaline the blood-sugar concentration rises to a maximum of over 150 mg./100 c.c. between the 30th and 60th minute, and does not regain its normal level until the lapse of 2-2½ hr. The respiratory exchange rises in both subjects and gradually falls again during the course of the experiment. As we found that the disturbance caused by the

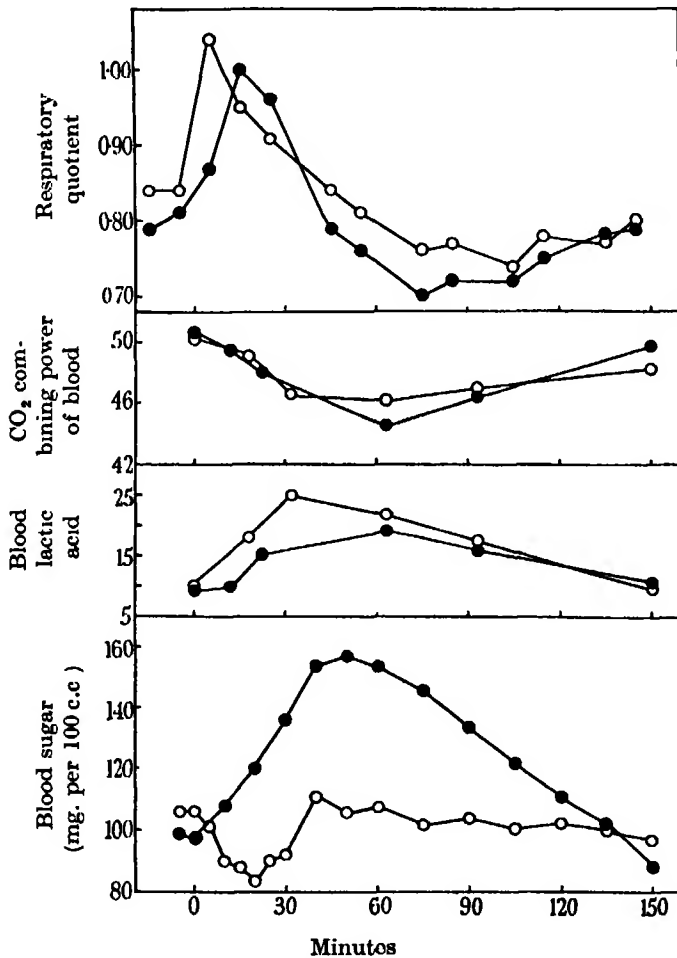


FIG. 7. Subject Douglas. Effect of adrenaline, and of adrenaline+insulin, on the blood sugar, blood lactic acid (mg./100 c.c.), CO<sub>2</sub> combining power of the blood at 40 mm. CO<sub>2</sub> pressure (in c.c. CO<sub>2</sub>/100 c.c.), and respiratory quotient. ● Adrenaline 0.5 mg. subcutaneously; ○ ditto + 2 units of insulin intravenously.

withdrawal of blood from a vein in the arm for lactic acid analyses etc. caused an obvious temporary increase in the respiratory exchange, we have in these experiments never collected expired air samples during the 10 min. immediately following the venepuncture so as to allow time for this disturbance to subside. The results of a typical experiment on each subject are shown in Table III.

The respiratory quotient shows a sharp initial rise which may reach

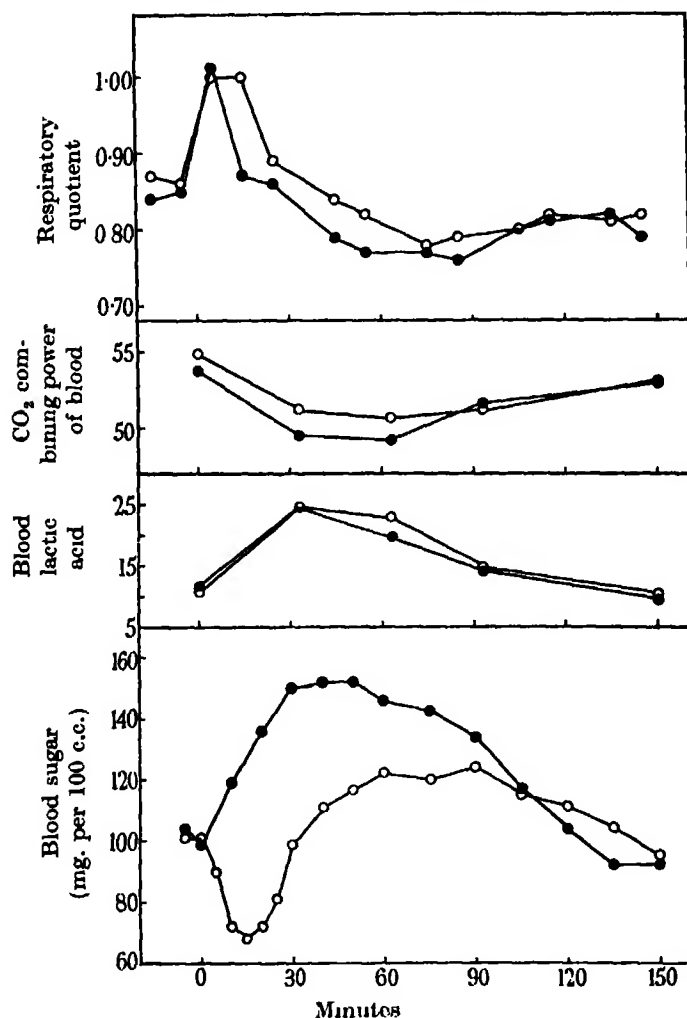


FIG. 8. Subject Courtice. Effect of adrenaline, and of adrenaline+insulin, on the blood sugar, blood lactic acid (mg./100 c.c.), CO<sub>2</sub> combining power of the blood at 40 mm. CO<sub>2</sub> pressure (in c.c. CO<sub>2</sub>/100 c.c.), and respiratory quotient. ● Adrenaline 0.5 mg. subcutaneously; ○ ditto + 1 unit of insulin intravenously.

TABLE III. AVERAGE RESPIRATORY EXCHANGE C.C./MIN.  
IN CONSECUTIVE PERIODS OF 10 MIN.

	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.
	Courtice			Douglas		
Before adrenaline	168	201	0.84	182	231	0.79
	185	218	0.85	187	232	0.81
After adrenaline	256	254	1.01	239	274	0.87
	229	263	0.87	259	258	1.00
	221	263	0.84	261	270	0.96
	—	—	—	—	—	—
	217	275	0.79	224	283	0.79
	201	261	0.77	208	281	0.74
	—	—	—	—	—	—
	201	260	0.77	190	270	0.70
	198	259	0.76	184	255	0.72
	—	—	—	—	—	—
	201	252	0.80	178	247	0.72
	206	256	0.81	176	236	0.75
	—	—	—	—	—	—
	198	241	0.82	163	210	0.78
	189	239	0.79	156	197	0.79

unity: this is succeeded by a fall to a level distinctly below the initial value, reaching a minimum in 1-1½ hr., followed by a gradual rise so that the initial value is often regained at the end of 2½ hr.

The lactic acid content of the blood rises considerably during the first half hour and subsequently decreases slowly to regain its initial value in 2½ hr.

The CO<sub>2</sub> combining power of the blood behaves as would be expected from the variation of lactic acid content, falling at first and then gradually rising again until it reaches, or nearly reaches, its initial level.

Figs. 7 and 8 also show the effect of the simultaneous injection of insulin intravenously and of adrenaline subcutaneously, the doses employed being 0.5 mg. adrenaline with 2 units of insulin for Douglas and 1 unit of insulin for Courtice. In fig. 7 (Douglas) the blood-sugar concentration shows an initial fall and recovery similar in time relations to the effect caused by the injection of insulin unaccompanied by adrenaline, save that the blood-sugar concentration falls to a minimum of only 80 mg./100 c.c. instead of 60. After this transitory depression the blood-sugar concentration remains practically unchanged at its initial level for the remainder of the experiment. In spite of the fact that there is no material increase in the blood-sugar concentration, the changes in the lactic acid concentration and the

CO<sub>2</sub> combining power of the blood, and in the respiratory quotient are practically the same as when adrenaline alone was injected, nor was there any difference in the behaviour of the oxygen consumption in the two cases. In fig. 8 (Courtice) there is the same transitory depression of the blood-sugar concentration, the lowest value reached being 70 mg./100 c.c. instead of 50–55 mg. which was found with insulin alone, and this is succeeded by a rise of the blood-sugar concentration to a level well below that found with adrenaline alone which slowly subsides. Here again there is no material difference in the behaviour of the lactic acid concentration, the CO<sub>2</sub> combining power of the blood or the respiratory quotient from that found when adrenaline was unaccompanied by insulin.

If 0.25 mg. adrenaline instead of 0.5 mg. were injected the results were of the same type as with the larger dose, the changes being merely on a smaller scale.

#### DISCUSSION

In these experiments the point that we had in view was the possible association of the changes of respiratory quotient during and after prolonged, though moderate, muscular work, which seem to be due to variations of carbohydrate metabolism, with alterations in the activity of the endocrine organs that can influence carbohydrate metabolism.

Taking the glucose tolerance tests as a whole it seems to us that the reduced tolerance which develops after prolonged exercise differs in no way from the reduced tolerance resulting from a diet poor in carbohydrate and rich in fat, and is due to similar causes. It is accompanied in both cases by a low respiratory quotient and ketonuria. Our present series of experiments therefore supports the conclusion, reached by Courtice and Douglas previously, that while it is impossible to exclude the conversion of fat to carbohydrate as a contributory cause of the persistent low respiratory quotient observed after prolonged exercise, by far the more important factor appears to be the diminution in the ratio of carbohydrate to fat oxidized, and the same factor may be expected when the proportion of carbohydrate to fat in the diet is reduced.

We cannot at present offer any explanation of the fact that if Courtice's glucose tolerance is tested immediately after muscular exercise the curve is almost the same as the normal one when there has been no preceding exercise, and there is clearly a delay before the characteristic reduction becomes evident. It may be due to the persistence of endocrine activity promoting carbohydrate metabolism during muscular work, but this is

hardly compatible with the fact that ketonuria develops during the first half-hour of the test. The matter seems to need more detailed investigation of the conditions during transition from work to rest.

The experiments described in this paper have been made on but two subjects, and we must emphasize the fact that the results are bound to vary with the individual. Mills has shown that he has quite a different type of tolerance curve, and that an amount of muscular exercise which is invariably followed by ketonuria in Courtice and Douglas causes none in him, nor does it cause any sensible alteration in his tolerance curve. These differences he ascribed, probably rightly, to the fact that his normal diet is rich in carbohydrate.

In spite of the reduction of glucose tolerance there was no evidence that the tissues were rendered less sensitive to an intravenous injection of insulin either by preceding exercise or, in Courtice's case, by a low-carbohydrate, high-fat diet, since the blood-sugar concentration fell at the same rate and to the same degree as in normal tolerance tests although its subsequent rise was slightly slowed.

Cori and Cori (1928) state that insulin causes no change in Calorie output but the isodynamic replacement of fat by carbohydrate oxidation. In following the respiratory exchange in detail we found but slight and inconstant effects on the oxygen consumption after small doses of insulin, on which we lay no stress, but there was invariably an increase in the respiratory quotient, more obvious in Courtice than in Douglas. Such an increase is only to be expected from the work of previous observers. In our own case we have argued above that this rise of quotient is probably determined by an increased metabolism of carbohydrate, and, tempting though it may be to suggest that the slight but maintained rise of quotient so often noted during continued muscular work of moderate severity may be dependent on a correlated increase in the secretion of insulin, there are difficulties to be faced before this view can be maintained. These difficulties involve not so much the actual magnitude of the change of quotient after a single intravenous injection of insulin as the time relations of this change and the fact that it is accompanied by a great fall in the blood-sugar concentration.

The latter difficulty must not however be overstressed for during muscular work other factors may help to combat the fall in blood-sugar concentration which would otherwise ensue from an increased secretion of insulin, and it may be relevant to point out that Christensen (1931) and Bøje (1936) have found that the untrained subject may show a fall in blood-sugar concentration in the earlier stages of continuous, though moderate, muscular



work before there is any question of fatigue, and that in Bøje's experiments this fall is accompanied by a rise in the respiratory quotient.

If a single intravenous injection of insulin is given to Courtice and Douglas whilst at rest the respiratory quotient does not begin to rise for some 15 and 8 min. respectively, it reaches a level which is maintained for a short time and then falls rather slowly. It may be thought that these changes are too slow in development and subsidence to explain the normal changes of quotient during moderate muscular work. Just after the beginning and cessation of muscular exercise it is difficult to assess the influence of an alteration in the proportion of carbohydrate to fat oxidized on the respiratory quotient since other factors are operative which themselves affect the quotient. We need only mention two of these, firstly the rise of body temperature that takes place when muscular work begins and the fall that ensues when work stops, and secondly the fact recently demonstrated so clearly by Bang (1936) that even in moderate muscular work excess lactic acid is formed in the first few minutes, presumably during the time required for the circulation and respiration to reach an equilibrium with the new condition of raised metabolism, but that if the muscular work is continued this excess may disappear so that a little later on we may find no more lactic acid in the blood than during normal rest.

An injection of insulin during actual muscular work shows, however, that the resultant effects develop more rapidly and to a greater degree than during rest, possibly because of the increase in the circulation rate and the alteration in the distribution of blood to the tissues. The effect on Courtice's blood-sugar concentration of an intravenous injection of one unit of insulin while he was doing steady work on the bicycle ergometer at the rate of 430 kg.m./min., involving an oxygen consumption of about 1000 c.c./min., is compared in fig. 9 with those of a normal experiment at rest (taken from fig. 4). The contrast between these two experiments is striking. After the same initial lag in the two cases the blood-sugar concentration falls far more sharply, and rises again much more rapidly, in the work than in the rest experiment. In the work experiment the blood-sugar concentration fell to 39 mg./100 c.c. at the 12th minute, and to 34 mg. at the 16th minute, as compared with the figure of 50 mg./100 c.c. at the 24th minute in the rest experiment. Hypoglycaemic symptoms were hardly appreciable at rest, but in the work experiment they were very severe in the form of profuse perspiration, faintness, disturbance of vision and diminution of hearing, which passed off fairly rapidly as the blood-sugar concentration rose again. Lawrence (1926) and Bürger and

Kramer (1928) drew attention to the fact that the fall of blood sugar after the subcutaneous injection of insulin in the diabetic is enhanced by muscular exercise, and this has been confirmed by general clinical experience. In the course of our experiments we gained a clear impression that a dose of insulin which causes no symptoms, or practically none, when the subject is at rest may cause quite perceptible symptoms if the subject moves about a little even though he does no active exercise.

Other endocrine secretions besides insulin influence carbohydrate metabolism, and adrenaline, with its power of mobilizing liver glycogen, is generally supposed to be an important factor during muscular work. We

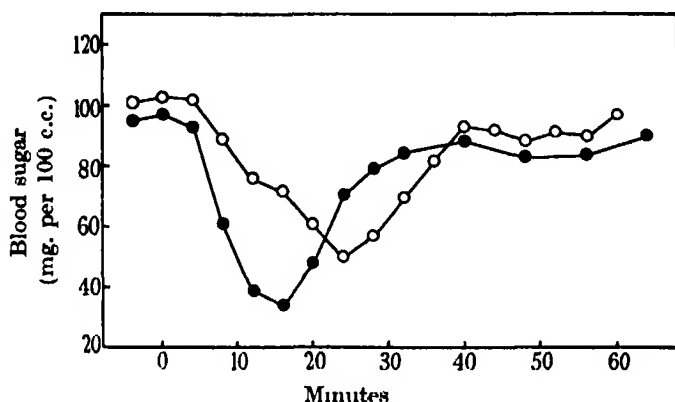


FIG. 9. Subject Courtice. Effect of 1 unit of insulin intravenously on the blood-sugar concentration. ○ At rest; ● during muscular exercise.

might therefore expect that insulin would have a more effective action on the respiratory quotient in the presence of increased adrenaline secretion.

Our own results are in complete agreement with the well established fact that adrenaline injection results in hyperglycaemia and excess lactic acid formation, as well as with the demonstration by Cori and others (1929, 1930) that if insulin is given simultaneously with adrenaline the rise of blood-sugar concentration that would occur with adrenaline alone may be greatly reduced, if not prevented entirely, without any significant change in the accumulation of excess lactic acid. Most other observers have found that adrenaline increases the metabolism. In our experiments the subcutaneous injection of adrenaline is constantly followed by an increased oxygen consumption which is evident for 2 hr. or longer, and is unaffected by the simultaneous injection of insulin. It is quite different from the inconstant results which we found with insulin alone. It is however the behaviour of the respiratory quotient that has principally interested us.

The changes in the respiratory quotient shown in our experiments after the injection of adrenaline alone the quick rise, the succeeding fall to a level below the resting normal, the final slow recovery—are characteristic, and entirely different from the change found after insulin injection. It is the classical picture which we have learnt to associate with the accumulation of excess lactic, or other non-volatile, acid in the blood, and the subsequent disappearance of this excess. It corresponds with the changes in lactic acid concentration and in the  $\text{CO}_2$  combining power of the blood, and a sufficient explanation can thus be offered to account for the facts.

When the subcutaneous injection of adrenaline is accompanied by the intravenous injection of insulin the changes of lactic acid concentration in the blood and of the respiratory quotient appear to be precisely the same as when adrenaline is injected alone, in spite of the fact that the rise of blood-sugar concentration is considerably reduced if not prevented, and the explanation of the change of quotient might well be the same in the two cases and depend merely on the formation and disappearance of excess lactic acid.

Calculation of the average respiratory quotient as shown in Table IV for the whole period of  $2\frac{1}{2}$  hr. after the injection of adrenaline or of

TABLE IV

	Average respiratory exchange c.c./min. before injection			Average respiratory exchange c.c./min. for entire period of $2\frac{1}{2}$ hr. after injection		
	$\text{CO}_2$	$\text{O}_2$	R.Q.	$\text{CO}_2$	$\text{O}_2$	R.Q.
Courtice						
0.5 mg. adrenaline	177	210	0.84	209	257	0.81
0.5 mg. adrenaline	169	201	0.84	197	239	0.82
0.5 mg. adrenaline + 1 unit insulin	176	204	0.86	212	252	0.84
Douglas						
0.5 mg. adrenaline	185	232	0.80	202	254	0.80
0.5 mg. adrenaline	182	227	0.80	210	267	0.79
0.5 mg. adrenaline + 2 units insulin	180	215	0.84	208	252	0.83

adrenaline + insulin shows that this, in Douglas's case, is practically identical in each instance with the preliminary quotient, and it looks as though the initial rise of quotient is exactly compensated by the subsequent fall. With Courtice the average quotient after the injection is rather lower than the preliminary figure. This might suggest that in these circumstances

there is no evidence from the changes of quotient of an increased oxidation of carbohydrate attributable to insulin, but that the variation of quotient depends solely on a lactic acid effect, and even that adrenaline exercises an inhibitory effect on carbohydrate oxidation which is otherwise promoted by insulin.

The experimental results do not however warrant so definite an attitude. When insulin is injected alone the rise of respiratory quotient is neither considerable nor long lasting. If an effect on the quotient of the same order as that shown in figs. 4-6 were also present in the earlier period of the experiments with adrenaline + insulin, it would be masked by the lactic acid effect on the quotient if this were calculated over so long a period as  $2\frac{1}{2}$  hr., since it might cause a change of no more than 0.01 in this average quotient. In spite of the striking similarity in the behaviour of the respiratory quotient after adrenaline alone and after adrenaline + insulin which suggests a common explanation, the possibility that insulin may contribute in minor degree to the rise of quotient cannot be excluded, although there is no indication that the adrenaline has substantially increased the effect of insulin on carbohydrate oxidation.

The hyperglycaemia and excess lactic acid formation seem to be two independent factors in the sense that insulin checks the former without materially affecting the latter. It might be thought that the advantage gained during muscular work by the mobilization of liver glycogen would be offset by the concurrent formation of excess lactic acid, but though lactic acid formation is the invariable accompaniment of such doses of adrenaline as we have used (Cori, Cori and Buchwald (1930) have found that if the dose of adrenaline is small enough there may be a slight rise in the blood-sugar concentration without a detectable change in the lactic-acid content of the blood. While therefore the mobilization of liver glycogen by adrenaline may be an important factor during muscular work, we have been unable to find any indication in our experiments during rest that the mere fact of such mobilization causes any significant increase in the ratio of carbohydrate to fat oxidized even when insulin is given at the same time.

Dill, Edwards and de Meio (1935) have found that a subcutaneous injection of 0.5-1.0 mg. of adrenaline during muscular work increases the respiratory quotient and the concentration of glucose and lactic acid in the blood. They conclude that the rise of quotient is mainly due to an increase in the proportion of carbohydrate oxidized, but in view of our own work we feel that a far more detailed analysis of the whole of the circumstances is required before such a conclusion can carry conviction.

## SUMMARY

1. Detailed observations have been made on two human subjects.
2. During rest after prolonged muscular work of moderate severity, when there was a persistent low respiratory quotient and ketonuria, there was a reduction of glucose tolerance which seems to be the same as that found without preceding exercise simply as a result of living on a low-carbohydrate, high-fat diet. This supports the conclusion that the main cause of the persistent low quotient after prolonged work is the low ratio of carbohydrate to fat oxidized.
3. The reduced glucose tolerance resulting from a low-carbohydrate, high-fat diet, as well as that shown after prolonged muscular work, was not accompanied by any significant difference in the response to insulin injected intravenously.
4. In the resting subject the temporary fall of blood-sugar concentration caused by intravenous injection of insulin was accompanied by a rise of the respiratory quotient which appeared to signify an increase of carbohydrate metabolism. There was no accumulation of excess lactic acid in the blood, and the effects on the oxygen consumption were slight and inconstant.
5. Subcutaneous injection of adrenaline caused an increase in the oxygen consumption. The respiratory quotient showed a characteristic change which could be explained by the accumulation and disappearance of excess lactic acid which accompanies the hyperglycaemia, without postulating any alteration in the oxidation of carbohydrate.
6. After the simultaneous injection of insulin and adrenaline the changes in blood-sugar concentration were diminished, but the changes of lactic acid concentration in the blood and of the respiratory quotient were practically identical with those after adrenaline alone.
7. The bearing of these experiments on the question of the possible significance of insulin and adrenaline secretion during muscular work is discussed.

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## The directional sensitivity of the retina and the spectral sensitivities of the rods and cones

BY W. S. STILES, *The National Physical Laboratory*

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### 1. INTRODUCTION

It is now well established that light rays of the same spectral character and physical intensity entering the eye through different points of the pupil may produce visual impressions which differ in brightness and colour even though the patch of retina stimulated (the fovea) is kept the same.\* Rays entering the eye through different points of the pupil and terminating on the same point of the retina are incident on the retina in different directions. Also, they have traversed different paths in the refractive media of the eye and may have suffered different losses by absorption, scattering or reflexion. It has been shown, however, that differences in the light losses in the refractive media do not account for the observed variations in visual response, which must therefore be attributed to variations in the reaction of the retina to light incident on it in different directions or, briefly, to a

\* See Stiles and Crawford (1933), Dziobek (1934), Wright and Nelson (1934), Goodlove (1936), Stiles (1937) and Crawford (1937).

*directional sensitivity* of the retina. Further evidence that this is so is given later in the paper (p. 81).

The first measurements of the directional sensitivity of the retina were made by photometric methods. The rays from one part of a photometric matching field entered the eye as a narrow pencil through a fixed point of the pupil. The point of entry of the pencil of rays from the other part could be varied. The intensity of this pencil could also be varied so as to bring the two parts to equality of brightness. Thus it was possible to determine the relative physical intensities of rays entering through different points of the pupil and producing the same subjective brightness. The *relative luminous efficiency*  $\eta$  of a ray entering through a particular point of the pupil was defined to be inversely proportional to the physical intensity required to produce a given subjective brightness. It was found (a) that the relative luminous efficiency is maximal for a certain point of entry  $P_M$  which is generally situated near the centre of the pupil, (b) that if  $\eta$  is put equal to unity for a ray entering at  $P_M$  then its value for a ray entering at a point  $P$  displaced  $r$  mm. from  $P_M$  is given approximately by the expression  $\eta = 10^{-pr^2}$ , where  $p$  is a constant equal to about 0.05, (c) that the value of  $p$  shows a small systematic variation with wave-length, being greatest in the blue, less in the red and least in the yellow. It was also found that the colour impression produced by monochromatic light of given wave-length varies as the point of entry of the ray is traversed across the pupil.

The results summarized above all refer to foveal vision of a photometric or colorimetric matching field. A different approach to the problem of retinal sensitivity is provided by the measurement of absolute thresholds and difference thresholds. In recent work a type of threshold measurement has been developed on the following lines. The eye views a given distribution of brightness (the conditioning stimulation) and at a given instant, an *additional* stimulus (the test stimulus) is applied over a given area of the field for a given time. The subject signifies whether or not the application of the test stimulus calls forth any visual impression. No analysis of the visual impression by the subject is required. He has not to decide, for example, whether he perceives a change of brightness or of colour. By successive trials using different intensities of the test stimulus, it is possible to determine the intensity at which the subject has a 50 % chance of perceiving the test stimulus. The intensity so obtained is termed the *liminal brightness increment* (l.b.i.). Usually the test stimulus is a small patch of light exposed for a brief interval of time. With the same conditioning stimulation, the variation of the l.b.i. with a property of the test

stimulus, such as its angular size, its exposure time or, for a monochromatic test stimulus, its wave-length, can be studied. In addition the effect of changes in the conditioning stimulation may be determined. For the purposes of this paper retinal sensitivity will be defined as the reciprocal of the l.b.i.

Measurements of the l.b.i. are much less precise than photometric measurements of brightness. On the other hand, the l.b.i. can be determined for any retinal area, foveal or extrafoveal, and the eye need not be in a steady state. The test stimulus will not in general disturb appreciably the state of adaptation of the retina. This is a valuable feature of the method, enabling us to determine, for example, the sensitivity of the retina to light of one wave-length when it is adapted to light of another wave-length.

In the present investigation the directional sensitivity of the retina has been studied by determining the variation of the l.b.i. as the position in the pupil of the point of entry of the rays constituting the test stimulus is varied. Both foveal and parafoveal vision of the test stimulus were employed. The conditioning stimulation consisted of a uniformly bright circular patch of light at the centre of which the test stimulus was applied. The case in which both test stimulus and conditioning stimulation are of white light has already been examined by Crawford (1937). In the present measurements, small bands of the spectrum were used for both test stimulus and conditioning stimulation. Crawford's results and those of the present paper show that the directional sensitivity of the parafoveal retina is very different for conditioning stimulations of high and low brightnesses respectively. This difference is here attributed to a transition from rod to cone vision as the brightness of the conditioning stimulation is raised. For many of the observations we are able to say whether the test stimulus was perceived by rod or by cone vision by considering the way in which the l.b.i. varies with the wave-length of the test stimulus and the intensity and wave-length of the conditioning stimulation.

The directional sensitivity of the foveal retina shows small but definite variations with the brightness of the conditioning stimulation which appear only for certain combinations of the wave-lengths of test stimulus and conditioning stimulation. These variations are attributed to transitions from vision by one type of cone to vision by another type. It proves possible to distinguish between perception of the test stimulus by different types of cone in foveal vision in much the same way as we distinguish between perception by rods and by cones in parafoveal vision. In the process, we obtain information about the spectral sensitivities of the different types of cone.



## 2. THE SUBJECT'S CONDITIONS OF OBSERVATION

The subject sat in a curtained enclosure and maintained his head in a fixed position by biting on a sealing-wax bit which was rigidly attached to the apparatus. He observed with one eye only, the other eye being covered with an eye shade. His field of view for parafoveal and foveal observation of the test stimulus is shown diagrammatically in figs. 1*a* and 1*b* respectively. For parafoveal observation, he directed his gaze at one of the two feeble points of light  $F_1$  and  $F_2$  (fixation points), the one not in use being removed from the field. For foveal observation, he looked towards the centre of the square of 3' side defined by the four feeble points of light  $N_1, N_2, N_3, N_4$  (orientation points). The test stimulus  $S$  appeared to him as a square patch of light of  $1.04^\circ$  side, exposed for 0.063 sec. at regular intervals once in every 3.6 sec. For foveal observation, the test stimulus appeared at the centre of the square  $N_1 N_2 N_3 N_4$  and for parafoveal observation at a point separated by  $5^\circ$  from the appropriate fixation point and in the position  $S$  shown in the figure. An audible signal, repeated just after each exposure, marked time for the subject. The subject held in his hand two keys, one of which he operated when he perceived the test stimulus and the other when he failed to do so.

The conditioning stimulation appeared to the subject as a uniformly bright and approximately circular patch of light of diameter  $10''$  (the central field  $C$ ), outside which the brightness of the field was zero. In an important special case the brightness of this central field was also zero. The test stimulus always appeared in the centre of the central field.

Prior to the commencement of a series of measurements the subject remained in the dark for a period up to 1 hr. depending on the nature of the measurements. The pupil of his observing eye was dilated when necessary by administering a few drops of a 5% solution of euphthalmine hydrochloride about 1 hr. before the measurements.

## 3. APPARATUS AND METHOD

The main principle of the apparatus is made clear by fig. 2. A parallel beam of light provided by spectrometer II is partially transmitted by the cube  $C$  and is brought to a focus  $\omega_2$  in the plane of the subject's pupil at  $O$  by the lens  $L_3$ . This beam is seen by the subject as a uniformly bright patch of light which forms the central field of the conditioning stimulation. A parallel beam from spectrometer I is delimited by a square aperture in the diaphragm  $T_3$ , is partially reflected at the diagonal surface in the cube  $C$

and is finally brought to a focus  $\omega_1$  in the plane of the subject's pupil by the lens  $L_3$ . The diaphragm  $T_3$  is so placed that the subject sees a virtual image of the square aperture at infinity. This image forms the test stimulus. It is important to note that in the area of the field of view occupied by the test stimulus, the eye receives radiation from both the test stimulus and the conditioning stimulation; thus in this sense, the test stimulus is added to or superposed on the conditioning stimulation. The fixation and orientation points are introduced from the side by reflexion in a thin plain glass plate  $M$  inserted in the parallel beam from spectrometer II. The rays forming a particular fixation or orientation point are reflected by  $M$  to form a real image in the plane  $K$  which is the focal plane of the lens  $L_3$ . This real image when seen through the lens  $L_3$  appears to the subject as a virtual image at infinity.

By slight rotations of the cube  $C$  the test stimulus beam can be sent into the eye through different points of the pupil, while the point of entry of the beam forming the central field remains practically unchanged. After such a rotation of cube  $C$ , the diaphragm  $T_3$  must be readjusted by a displacement in its own plane to restore the test stimulus to its original position in the subject's field of view.

A diagram of the complete apparatus is shown in fig. 3. As the apparatus differs little from that described in a previous paper (Stiles 1937), it will suffice to indicate its main features and such changes and additions as have been made.

Images of the ribbon filament source  $S_1$  are formed on the entrance slits of the two spectrometers I and II. The rays from the exit slit of spectrometer I pass through the continuous wedge  $W_1$  and the step-wedge  $W_2$  and are rendered parallel by the lens  $L_2$ . The rays from the exit slit of spectrometer II pass through the continuous wedge  $W_3$  and are rendered parallel by lens  $L_4$ . The two parallel beams from the spectrometers are then focused to give images  $\omega_1$  and  $\omega_2$  of the respective exit slits at the subject's eye, as already explained. The auxiliary optical system  $L_7-G-E$  enables the relative positions of the slit images to be determined without disturbing the subject.

At  $R_1$  a shutter is interposed in the beam of spectrometer I. The shutter consists of two rotating disks geared together so that one rotates three times for each rotation of the other. There is an indentation in the periphery of each disk and light is passed by the shutter only when the two indentations come into coincidence opposite the entrance slit of the spectrometer. The width of the indentation in the faster disk, the radius of this disk and its speed of rotation determine the exposure time of the test

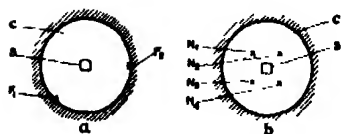


FIG. 1. Subject's field of view (a) for parafoveal, (b) for foveal observation.

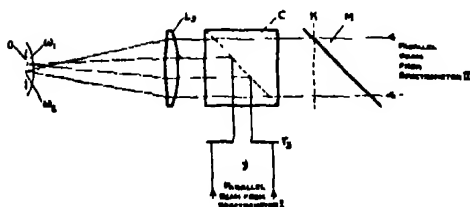


FIG. 2. Diagram showing the principle of the measurements.

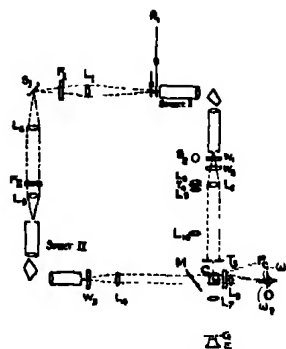


FIG. 3. Diagram of the apparatus (not to scale).

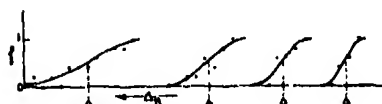
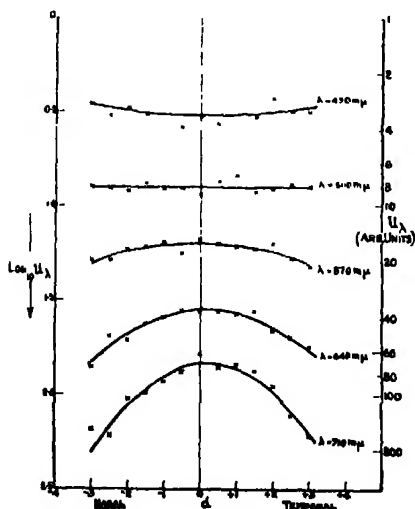


FIG. 4. Derivation of  $\Delta$ .



stimulus. The use of two disks in this way, rather than a single disk, gives a shutter of higher efficiency. The slower disk carries contacts operating a buzzer once in every 3.6 sec.

The thread controlling the position of the step wedge  $W_2$  is connected to a carriage moving on rails across a wide strip of recording paper which is given a slow motion in the direction of its length. Two pens, marking in different colours and operated by the keys in the subject's hands, are fixed to the carriage. Between successive exposures of the test stimulus the carriage is moved to bring different steps of the wedge  $W_2$  into the beam so that on the paper a record is obtained of the subject's responses to different intensities of the test stimulus.

Stray light filters and neutral filters can be inserted in the beam of spectrometer I, of spectrometer II or in the common beam at  $F_1$ ,  $F_{11}$ ,  $F_c$  respectively.

The fixation or orientation points are obtained in the following manner. An opal lamp  $S_2$  placed at the focus of lens  $L_8$  is imaged at the focus of the similar lens  $L_9$ , the image being at the focus of lens  $L_{10}$ , so that finally an image of the lamp is formed on the eye by the lens  $L_3$ . Between lenses  $L_8$  and  $L_9$  a metal diaphragm  $T_4$  is inserted containing one or four small holes (fixation or orientation points respectively). The images of these holes formed by lens  $L_{10}$  are arranged to lie in the focal plane of lens  $L_3$  (allowing for reflection in  $M$ ) so that the subject sees the fixation or orientation points at infinity. A resistance enables the subject to adjust the intensity of the lamp  $S_2$  and colour filters can be inserted in the beam if required.

The positioning of the eye with respect to the slit images  $\omega_1$  and  $\omega_2$  and the measurement of the separation of  $\omega_1$  and  $\omega_2$  were carried out by methods similar to those previously described. When filters were inserted at  $F_c$ , a slight displacement of the images  $\omega_1$  and  $\omega_2$  usually occurred. The images were restored to their correct positions by a suitable shift of the lens  $L_3$  in its own plane.

The method of calculating the intensities of the areas of the subject's field of view illuminated by the two spectrometers was the same as in the previous investigation. The calculations yield the values of the following quantities:

$U'_\lambda$  = the flux of radiant energy of wave-length  $\lambda$ , expressed in ergs/sec., received by the eye from spectrometer I during the exposure of the test stimulus, *divided by* the angular area of the test stimulus expressed in square degrees. (For brevity  $U'_\lambda$  will be referred to as the "energy" of the test stimulus.)

$W_\mu$  = the flux of radiant energy of wave-length  $\mu$ , expressed in ergs/sec., received by the eye from spectrometer II *divided by* the area in square degrees of the field illuminated by spectrometer II. ( $W_\mu$  will be referred to as the energy of the central field.)

Corresponding to  $U'_\lambda$  and  $W_\mu$ , the conventional photometric brightnesses  $B'_U$  and  $B_W$  of the test stimulus and central field are derived from the relations:

$$B'_U = 2.02 \times 10^3 U'_\lambda V_\lambda,$$

$$B_W = 2.02 \times 10^3 W_\mu V_\mu,$$

where  $V_\lambda$  and  $V_\mu$  are the values of the standard relative luminosity factor for wave-lengths  $\lambda$  and  $\mu$  respectively.  $B'_U$  is defined as the brightness in candles per square foot which, if viewed through an artificial pupil of area 10 sq. mm. by an eye possessing the standard curve of relative luminosity, would match in brightness the test stimulus as seen by a similar eye in the actual apparatus.  $B_W$  is similarly defined.\*

The spectral purity of the light in test stimulus or central field is specified by the wave-length difference  $\Delta\lambda$  or  $\Delta\mu$  between the extreme limits of the spectrum band passed by the spectrometer. In general,  $\Delta\lambda$  and  $\Delta\mu$  had values of the order of 15 and 30 m $\mu$  respectively. Parasitic light of wave-length lying outside the desired band was reduced to an unimportant amount by the use of coloured stray-light filters.

For the exit slit of spectrometer I a fixed slit of height 0.50 mm. and width 0.48 mm. was used for all the measurements. The image  $\omega_1$  of this slit had the same dimensions. For the entrance slit, fixed slits of various widths were used. The slit height of spectrometer II was either approx. 0.50 or 2.20 mm. The continuously variable symmetrical slits were adjusted as required.

The calculations of  $U'_\lambda$  and  $W_\mu$  were made assuming the slit widths so small that slit-width corrections could be neglected.

#### 4. DERIVATION OF THE LIMINAL BRIGHTNESS INCREMENT FROM THE RECORD OF THE SUBJECT'S RESPONSES

The logarithm of the energy of the test stimulus during a measurement of the l.b.i. may be expressed in the form

$$\log_{10} U'_\lambda = \log_{10} (U'_\lambda)_0 - \Delta_n,$$

\*  $B'_U$  and  $B_W$  can be obtained in terms of photons by replacing the factor  $2.02 \times 10^3$  by  $2.18 \times 10^5$ .

where  $(U'_\lambda)_0$  is the energy when the step wedge  $W_2$  was removed from the beam the other conditions remaining unchanged, and  $\Delta_n$  is the optical density for wave-length  $\lambda$  of the  $n$ th step of wedge  $W_2$ . If the subject recorded "seen"  $M$  times, and "not seen"  $N-M$  times when a particular step of  $W_2$  was in the beam, then  $f = M/N$  may be regarded as an approximation to the probability of seeing the test stimulus for that particular energy. Determinations of  $f$  for a series of consecutive values of  $n$  gave a graph of  $f$  against  $\Delta_n$  from which the l.b.i. could be derived. Examples of such graphs are shown in fig. 4. In each case five exposures were made at each step, i.e.  $N = 5$ . The density difference between consecutive steps equalled approximately 0.07 but varied slightly with wave-length. The S-shaped curves drawn in fig. 4 are all of the form:

$$\begin{aligned} f &= \frac{1}{2}[1 - P\{h(\Delta_n - \Delta)\}] \text{ for } \Delta_n > \Delta \\ &= \frac{1}{2}[1 + P\{h(\Delta_n - \Delta)\}] \text{ for } \Delta_n < \Delta, \end{aligned}$$

in which  $\Delta_n$  is allowed to vary continuously,  $\Delta$  and  $h$  are constants, and  $P\{x\}$  is the probability integral  $\frac{2}{\sqrt{\pi}} \int_0^x e^{-x^2} dx$ . A relation between  $f$  and  $\Delta_n$  of this form is to be expected if the following conditions hold:

(i) when the test stimulus is exposed there is a critical intensity which must be exceeded if the subject is to record "seen",

(ii) the critical intensity varies from exposure to exposure in a random manner about an average value corresponding to a density in the beam equal to  $\Delta$ ,

(iii) if  $\Delta'$ ,  $\Delta''$ ,  $\Delta'''$ , etc. are the densities corresponding to the critical intensities at the instants of different exposures, then  $\Delta'$ ,  $\Delta''$ ,  $\Delta'''$ , etc., form a Gaussian distribution about a mean value  $\Delta$  with a modulus of precision  $h$ .

In most cases an S-shaped curve of the above form could be fitted to the plot of  $f$  against  $\Delta_n$  with the measure of agreement illustrated in fig. 4.

In practice, tracings of the theoretical S-shaped curve corresponding to different values of  $h$  were prepared. The traced curves could be placed in turn over the experimental graph of  $f$  against  $\Delta_n$  and the pair of values of  $h$  and  $\Delta$  giving the best fit could be determined "by inspection".

The energy  $U_\lambda$  of the test stimulus corresponding to the density value  $\Delta$  is given by the relation

$$\log_{10} U_\lambda = \log_{10} (U'_\lambda)_0 - \Delta.$$

$U_\lambda$  is the energy of the test stimulus at which the subject's response is as likely to be "seen" as "not seen".  $U_\lambda$  is accepted as the value of the l.b.i. yielded by the measurement. Corresponding to each determination of  $U_\lambda$

we obtain in  $h$  an estimate of the sharpness of the liminal value, or of the extent to which the critical intensity referred to in (i) above varies from exposure to exposure.

In the first measurements a determination of the l.b.i. was based on sixty exposures, made up of five exposures each of nine consecutive steps of wedge  $W_2$ , which were known from a preliminary rough setting by the subject to embrace the liminal value, together with fifteen blanks (test stimuli of zero intensity). The sixty exposures were presented in random order. It was soon found that a "seen" response to a blank was obtained only very rarely. For the bulk of the measurements the stimuli were presented in order of decreasing or increasing intensity, the step wedge being moved one step after each exposure. Each succession of exposures covered the range from "certainly seen" to "certainly not seen" or vice versa. Three successions of exposures with increasing intensity and two with decreasing intensity completed the measurement. No blanks were included. Tests showed that this method gave about the same values of the l.b.i. as the first method but the precision was a little better; it imposed less strain on the subject and called for a less precise preliminary setting. It was adhered to for all the subsequent measurements.

##### 5. SPECIFICATION OF THE DIRECTION OF INCIDENCE OF A RAY ON THE RETINA

The direction of incidence on the retina is the effective variable determining the change in retinal response when a ray enters the eye through different points of the pupil. As in previous work, this direction will be specified indirectly by giving the position of the point of entry of the ray in the pupil or rather in the apparent pupil, i.e. the pupil seen from outside the eye. The ray entering through the centre of the apparent pupil, and terminating on a given retinal point may be regarded as defining the normal to the retina at that point. Any other ray entering the apparent pupil at a point  $d$  mm. from the centre and terminating on the same retinal point will strike the retina at an angle of incidence (i.e. at an angle with the normal ray) equal to  $2.4d$  deg. This result is only a rough approximation applicable for an average normal eye and for retinal points at or near the fovea. Since for the fully dilated pupil  $d$  may have a value up to about 4 mm., we are dealing with the effects of changing the angle of incidence from  $0^\circ$  to about  $10^\circ$ .

There are practical advantages in keeping the point of entry on the horizontal diameter of the apparent pupil and this has been done in all the

present measurements. Thus we require only one variable, namely  $d$ , the distance in mm. from the point of entry to the centre of the apparent pupil,  $d$  is taken as positive when the point of entry is on the temporal and negative when it is on the nasal side of the centre. It is easy to show that when the relative luminous efficiency  $\eta$  can be represented by the expression  $10^{-pr^2}$ , where  $r$  is the distance in mm. of the actual point of entry from the point of entry  $P_H$  giving maximal luminous efficiency, then the variation of  $\eta$  across the horizontal diameter is represented by the analogous expression  $10^{-p(d-d_m)^2}$ , where  $p$  has the same value as before and  $d_m$  defines the position of the point of entry which gives a greater luminous efficiency than any other point on the horizontal diameter.

The pupil is not a fixed structure in the eye. Its diameter and, possibly, the position of its centre change with the lighting conditions. It appears from the present measurements that, when dilated with a mydriatic, the pupil does not always assume the same diameter. The centre of the dilated pupil has been found to shift slightly ( $\frac{1}{10}$  to  $\frac{2}{10}$  mm.) during the course of a series of measurements. These vagaries are unfortunate for the present purpose.\*

## 6. RESULTS. CENTRAL FIELD OF ZERO BRIGHTNESS

All the measurements reported refer to the writer's left eye. This is a slightly hypermetropic eye (+0.5 Sph. -0.25 Cyl. 130°) and a normal trichromat belonging to Abney and Watson's Class I (rod-free fovea). The examination of other eyes was deferred until the results of the present work should have indicated the points meriting special study.

We consider first the case in which the subject's field of view was completely dark save for the fixation or orientation points and the flashes of the test stimulus. Prior to every series of measurements the subject remained for about 1 hr. in the dark. Thus the observations were made with the dark-adapted eye. Fig. 5 shows, for five wave-lengths of the test stimulus, the variation of the logarithm of the l.b.i. for 5° parafoveal vision (fixation point  $F_1$ ) as the point of entry of the rays was moved across the horizontal diameter of the apparent pupil. Each plotted point is the mean of two or more values obtained in independent runs. In each run half the points were obtained in a traverse from nasal to temporal, the other half in a traverse in the reverse sense. At present we are concerned only with the

\* A method of specifying point of entry independently of the pupil has been suggested to the writer by Professor Hartridge. The proposal is to use as a fixed point of reference in the eye the reflected image in the cornea of a distant light on which the subject fixates.



variation of  $\log_{10} U_\lambda$  and an arbitrary constant has been added to the absolute values for each curve to give a convenient spacing of the different curves in the figure. In the figure the scale of  $\log_{10} U_\lambda$  has been taken to be increasing in the downwards direction, so that the curves represent the variation of  $(-\log_{10} U_\lambda) = \log_{10} 1/U_\lambda$  with respect to a similar scale increasing in the upwards direction. The curves show that the sensitivity of the dark-adapted parafoveal retina is practically non-directional for test stimuli in the blue-green but exhibits a slight directional effect in the yellow, which becomes well defined in passing through orange to red. In the violet there appears to be a slight effect in the reverse sense.

Similar measurements for foveal vision of the test stimulus are given in fig. 6. The familiar difficulties in measuring the foveal threshold (save in the red end of the spectrum), due to the greater sensitivity of the parafovea, are accentuated when the rays enter near the edge of the pupil. This is so because the parafoveal sensitivity is maintained while the foveal sensitivity falls off as the point of entry moves from centre to periphery of the pupil.

Fortunately with practice the subject can tell from the appearance of the test stimulus whether his fixation has wandered so that he is using parafoveal instead of foveal vision. When this occurred, the subject operated neither key and the exposure was repeated. It sometimes happened when the test stimulus was correctly fixated, that the light scattered or irregularly reflected in the eye could be seen parafoveally although the normal image of the test stimulus produced no response. This was not accepted as vision of the test stimulus.

The curves of fig. 6 show that the sensitivity of the dark-adapted foveal retina exhibits a pronounced directional effect whatever the wave-length of the test stimulus.

It is appropriate to compare the variation of  $\log_{10}(1/U_\lambda)$  with point of entry and the corresponding variation of  $\log_{10} \eta$ , where  $\eta$  is the relative luminous efficiency obtained in brightness matching measurements. The variation of  $\log_{10}(1/U_\lambda)$  can in fact be represented to a first approximation by the empirical formula:

$$\log_{10}(1/U_\lambda) = \log_{10}(1/U_\lambda)_m - p_\lambda(d - d_m)^2, \quad (1)$$

where  $\log_{10}(1/U_\lambda)_m$  is the maximum value of  $\log_{10}(1/U_\lambda)$  which is attained at  $d = d_m$ , and  $p_\lambda$  is a constant. This formula is of the same form as that previously found for  $\log_{10} \eta$  and there can be little doubt that the same cause is operative in the two types of experiment. The continuous curves drawn in figs. 5 and 6 have been computed from the above formula with suitable

choice of the constants  $p_\lambda$  and  $d_m$ . For the foveal measurements, the data show a tendency (very marked for  $\lambda = 430 \text{ m}\mu$ ) to deviate from the fitted curve for  $(d - d_m)$  greater than 3 mm. A similar deviation was observed in the curves of  $\log_{10} \eta$  against  $d$ . The values of  $p_\lambda$  for foveal vision come out to be about 15 % lower than those derived from the  $\log_{10} \eta$  curves obtained for the same eye in the brightness matching experiments with monochromatic light (Stiles 1937). The position of the maximum  $d_m$  is also displaced from about 0.6 mm. temporal to about 0.9 mm. temporal.

To see whether these differences were due to a difference in the luminous efficiencies operative for the threshold measurements and for the brightness matching measurements, or to an actual change in the eye, brightness matching traverses were made for  $\lambda = 500 \text{ m}\mu$  and for  $\lambda = 700 \text{ m}\mu$  (foveal vision). The results gave values of  $p_\lambda$  and  $d_m$  in substantial agreement with those found from the corresponding threshold curves. It must be concluded that for this eye the direction of incidence on the retina of the ray giving maximal luminous effect and the magnitude of the directional effect specified by  $p_\lambda$  have changed. Changes of the same kind are in evidence if the measurements of  $\eta$  made with monochromatic light (Stiles 1937) are compared with the original measurements made with white light (Stiles and Crawford 1933). It appears that in the course of about six years the point of entry in the horizontal diameter giving the greatest value of  $\eta$  has shifted for this eye from about 0.2 mm. nasal to about 1.0 mm. temporal. Whatever the actual mechanism giving rise to the directional properties of the retina, we are probably justified in regarding the direction of incidence which gives the greatest value of  $\eta$  as defining in some manner the direction in which the end organs of the retina are pointing. It is possible therefore that for the eye in question the end organs at the fovea are being gradually sheared over. During about the same period of time the refraction of the eye has changed from emmetropic to +0.5 Sph. -0.25 Cyl. 130° but no other symptom which might possibly be correlated with the changes in  $d_m$  has been noted.

The values of  $d_m$  for several eyes studied by Crawford and the writer are given in Table I. In some cases the point of entry was varied on the vertical diameter of the pupil and  $d_m$  then defines the position on the vertical diameter giving the greatest value of  $\eta$ . The values of  $d_m$  for extrafoveal points were obtained from measurements of the l.b.i.

Unfortunately the pupil centre is not a perfectly stable reference point and the value of  $d_m$  is subject to some ambiguity on this account. However, the figures in the table show that  $d_m$  varies appreciably for different eyes, and for different retinal points of the same eye. The systematic

TABLE I. POSITION OF THE POINT OF ENTRY ON THE HORIZONTAL OR VERTICAL DIAMETER OF THE PUPIL OF THE RAY GIVING THE GREATEST VISUAL EFFECT

Subject	Date of measurement and reference		Method of measurement	Retinal patch	Diam. of pupil traversed	$d_m$ in mm.
L.E. = left eye, R.E. = right eye						
W.S.S. (L.E.)	1932	(1)	Brightness matching	Fovea	Hor.	-0.2 (nasal)
"	1932	(1)	"	"	Vert.	-0.5 (upper)
"	1932*	(1)	"	"	Hor.	0
"	1932	(1)	"	"	Vert.	-0.2 (upper)
"	1936	(2)	"	"	Hor.	+0.6 (temp.)
"	1936	(2)	"	"	Vert.	-0.5 (upper)
"	1937-8	(4)	"	"	Hor.	+0.9 (temp.)
"	1937-8	(4)	l.b.i. measurements	"	"	+0.9 (temp.)
"	1937-8	(4)	"	5° parafovea (fixation point $F_1$ )	"	+0.2 (temp.)
"	1937-8	(4)	"	5° parafovea (fixation point $F_2$ )	"	+1.5 (temp.)
B.H.C. (L.E.)	1932	(1)	Brightness matching	Fovea	"	-0.5 (nasal)
"	1932	(1)	"	"	Vert.	+0.6 (lower)
B.H.C. (R.E.)	1932	(1)	"	"	Hor.	-0.5 (nasal)
"	1932	(1)	"	"	Vert.	+0.2 (lower)
B.H.C. (L.E.)	1937	(3)	l.b.i. measurements	"	Hor.	-0.5 (nasal)
"	1937	(3)	"	5° parafovea	"	-1.5 (nasal)
"	1937	(3)	"	14° parafovea	"	-1.7 (nasal)
R.H.S. (L.E.)	1937	(3)	"	Fovea	"	-1.4 (nasal)
"	1937	(3)	"	5° parafovea	"	-1.8 (nasal)
F.W.C. (R.E.)	1932	(1)	Brightness matching	Fovea	"	+0.5 (temp.)
"	1932	(1)	"	"	Vert.	0

\* Six weeks later.

References: (1) Stiles and Crawford (1933); (2) Stiles (1937); (3) Crawford (1937); (4) Stiles (present work).

determination of  $d_m$  in the vertical and horizontal diameters for a large number of eyes would be of considerable interest.

Further measurements of the effect of wave-length on the directional sensitivity were made by the *two-point method*, in which  $\log_{10} U_\lambda$  is determined for two points of entry,  $P_n$ , at or near the point giving maximal sensitivity (central entry) and  $P_p$ , near the periphery of the pupil (peripheral entry). Measurements were made for a series of wave-lengths spanning the spectrum in one run. From the two values of  $\log_{10} U_\lambda$  obtained for

each wave-length and from the co-ordinates  $d_o$  and  $d_p$  of the points  $P_o$  and  $P_p$ , the value of  $p_\lambda$  was computed using formula (1). It was necessary to assume the value of  $d_m$  obtained earlier from the curves of figs. 5 and 6.

Figs. 7 and 8 give the mean values of  $p_\lambda$  obtained from five two-point determinations for parafoveal vision and from three such runs for foveal vision. For parafoveal vision,  $p_\lambda$  is not very different from zero for  $\lambda$  less than 600 m $\mu$ . As  $\lambda$  increases from 600 to 700 m $\mu$ ,  $p_\lambda$  increases to approximately 0.045 and finally it appears to drop again slightly. For foveal vision  $p_\lambda$  lies in the range 0.045 to 0.065 and shows a variation with wave-length similar to that found previously in the brightness-matching experiments. The values of  $p_\lambda$  obtained from new measurements by the brightness-matching method (two point determinations) are shown as the circles in fig. 9 which also gives the values obtained in the earlier investigation. Apart from the shift in absolute value already noted, the new determinations of  $p_\lambda$  by the brightness-matching method show the same variation with wave-length as the old and this variation is closely followed by the values of  $p_\lambda$  derived from the l.b.i. determinations.

Determinations of  $p_\lambda$  at a second parafoveal point, again at  $5^\circ$  from the fovea, but in a different meridian, are plotted in fig. 10. For these measurements the fixation point  $F'_2$  was used. Save for the point at  $\lambda = 450$  m $\mu$ ,  $p_\lambda$  shows a similar variation with wave-length to that obtained for the other parafoveal point. For this parafoveal point,  $d_m$  had the value 1.5 mm. (temporal).

We may sum up the above results as follows. the sensitivity of the dark-adapted parafovea is nearly independent of the direction of incidence of the light on the retina except for wave-lengths in the orange and red. The sensitivity of the dark-adapted fovea shows a pronounced variation with direction of incidence for all wave-lengths. The magnitude of the directional effect for the fovea varies to a limited extent with wave-length and the variation resembles that found in the earlier experiments by the brightness-matching method.

In parafoveal observation of a coloured test stimulus, the fact that the test stimulus is coloured can be appreciated only when its intensity exceeds a certain multiple of the threshold value. This multiple, known as the photochromatic ratio, was observed to be greater for peripheral entry than for central entry of the light rays in the pupil. For example, at 700 m $\mu$  it had on the average the value 1.4 for central, and 2.5 for peripheral entry. At shorter wave-lengths, the values were of course very much larger but showed a difference in the same sense between central and peripheral entry.

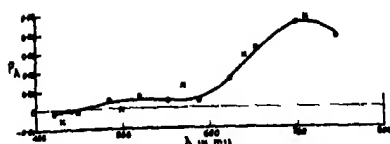


FIG. 7. Variation of  $p_\lambda$  with  $\lambda$  for the dark-adapted parafovea ( $F_1$ ).  $\odot$ , Two-point determinations;  $\times$ , from data of fig. 5.

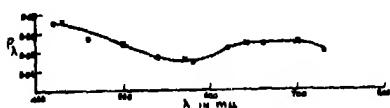


FIG. 8. Variation of  $p_\lambda$  with  $\lambda$  for the dark-adapted fovea.  $\odot$ , Two-point determinations;  $\times$ , from data of fig. 6.

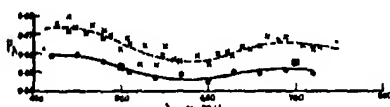


FIG. 9. Variation of  $p_\lambda$  with  $\lambda$  by brightness-matching method.  $\odot$ , New values by two-point method;  $\square$ , new values from traverses;  $\times$ , old values, Stiles (1937).



FIG. 10. Variation of  $p_\lambda$  with  $\lambda$  for the dark-adapted parafovea ( $F_2$ ).  $\odot$ , Two-point method (single determination);  $\square$ , from traverses.

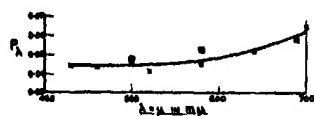


FIG. 13. Variation of  $p_\lambda$  with  $\lambda$  for the light-adapted parafovea ( $F_1$ ).  $\lambda = \mu$ ;  $\times$ , Single determination by two-point method;  $\log_{10} W_\mu = 3.2$  (500 mμ), 3.6 (580 mμ), 2.7 (690 mμ).  $\square$ , From traverses of fig. 12.

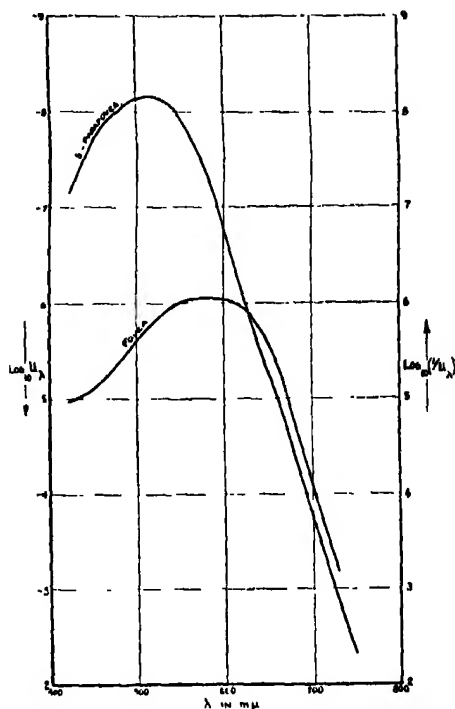


FIG. 11. Spectral sensitivities of dark-adapted fovea and parafovea ( $F_1$ ). Central entry.

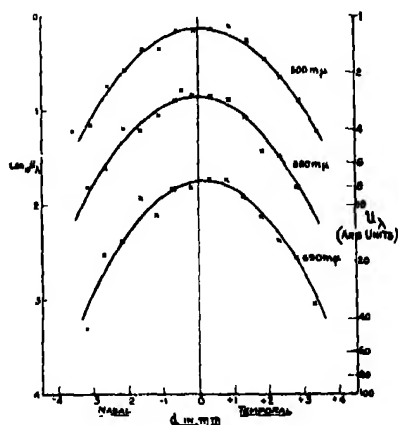


FIG. 12. Directional sensitivity of the light-adapted parafovea ( $F_1$ ).  $\lambda = \mu$ ;  $\log_{10} W_\mu = 3.2$  (500 mμ), 3.6 (580 mμ), 2.7 (690 mμ).

In foveal observation of the test stimulus, the photochromatic ratio does not depend on the point of entry and, in fact, it has the value 1 for all colours except yellow.

The mean values of  $\log_{10} U_\lambda$  for central entry, obtained in the two-point determinations, are plotted against wave-length in fig. 11. The actual values are given and not the values plus an arbitrary constant as in figs. 5 and 6. The curves of fig. 11 are of the type first obtained by Abney and Watson (1916) and they can be interpreted in terms of the duplicity theory of vision in the following manner. Foveal vision of the test stimulus is served solely by the cones since rods are absent from the fovea. Thus the foveal curve represents the variation with wave-length of the logarithm of the l.b.i. of the foveal cone system. The parafovea contains both rods and cones. For any wave-length of the test stimulus the parafoveal rods acting alone would give one value of the l.b.i.,  $(U_\lambda)_r$ , and the parafoveal cones acting alone would give another value  $(U_\lambda)_p$ . The l.b.i. of the parafoveal cones may be assumed to vary with wave-length in much the same way as the l.b.i. of the foveal cones so that the graph of  $\log_{10}(U_\lambda)_p$  against  $\lambda$  will have the same form as the foveal curve of fig. 11 although it may be displaced parallel to the axis of  $\log_{10} U_\lambda$ . In reality the rods and cones are in action together and although we do not know how their respective effects "add up" we may safely assume that the resultant parafoveal l.b.i. will not be greater than either  $(U_\lambda)_r$  or  $(U_\lambda)_p$ . Thus since for  $\lambda$  greater than 630 m $\mu$  the parafoveal l.b.i. exceeds the foveal l.b.i., it follows *a fortiori* that  $(U_\lambda)_p$  will exceed the foveal l.b.i. This means that the parafoveal cone system is less sensitive than the foveal cone system. On the other hand for  $\lambda$  less than 600 m $\mu$ , the parafoveal l.b.i. is much smaller than the foveal l.b.i. and, *a fortiori*, much smaller than  $(U_\lambda)_p$ . Thus in this region of the spectrum we can identify the parafoveal l.b.i. with  $(U_\lambda)_r$ , the l.b.i. of the parafoveal rod system. As  $\lambda$  increases beyond 600 m $\mu$  it is probable that the ratio of  $(U_\lambda)_p$  to  $(U_\lambda)_r$  tends to diminish, until at 700 m $\mu$  the two quantities are not very different. This is suggested by the fact that the photochromatic ratio for parafoveal vision diminishes for  $\lambda$  greater than 600 m $\mu$ , until at  $\lambda = 700$  m $\mu$  it equals approximately 1.4. Thus, the parafoveal cones may participate to an increasing degree in determining the parafoveal l.b.i. as we pass from 600 m $\mu$  to the extreme red end of the spectrum.

If we accept the above interpretation, then in fig. 8 the values of  $p_\lambda$  measure the directional sensitivity of the foveal cones. In fig. 7 the values of  $p_\lambda$  for  $\lambda$  less than 600 m $\mu$  measure the directional sensitivity of the parafoveal rods. The increase in  $p_\lambda$  at longer wave-lengths may be due (a) to an increase in the directional sensitivity of the parafoveal rods with increase

in wave-length, (b) to the participation of the parafoveal cones which, like the foveal cones, may show a large directional sensitivity at all wave-lengths, (c) to a combination of these two causes.

## 7. CENTRAL FIELD ILLUMINATED: PARAFOVEA

For all the observations described in this section, the light rays forming the central field entered the subject's eye through the centre of the pupil. The test stimulus was observed parafoveally using fixation point  $F_1$ . With a central field of high brightness and of the same wave-length as the test stimulus, the variation of  $\log_{10} U_\lambda$  with point of entry of the test stimulus was determined for wave-lengths 500, 580 and 690 m $\mu$  (fig. 12). The values of  $p_\lambda$  obtained from two-point determinations under similar conditions are plotted against  $\lambda$  in fig. 13. These results show that when the parafoveal retina is adapted to a high brightness of given wave-length, it exhibits a well-developed directional sensitivity to a test stimulus of the same wave-length whatever that wave-length may be.

The differences in the directional sensitivities of the light- and dark-adapted parafoveal retina, which are evident from a comparison of figs. 5 and 12 or 7 and 13, prove conclusively that we are in fact dealing with the directional properties of the retina and not with differences in light losses in the refractive system of the eye. Effects of the latter kind would be unaffected by the presence or absence of the central field.

By determining  $\log_{10} U_\lambda$  for central and peripheral entry of the test stimulus at a series of brightnesses of the central field from zero upwards the change in directional sensitivity with brightness level could be followed. The subject remained in the dark for 1 hr. before each run, his pupil having been previously dilated with euphthalmine. The mean results of two runs at each of the wave-lengths 500, 580 and 690 m $\mu$  are plotted in figs. 14, 15 and 16. Each figure gives three curves:

- I.  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  for central entry of the test stimulus.
- II.  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  for peripheral entry of the test stimulus.
- III.  $p_\lambda$  against  $\log_{10} W_\mu$  derived from the previous curves by applying formula (1).

Consider first fig. 15 for which  $\lambda = \mu = 580$  m $\mu$ . The curve of  $p_\lambda$  against  $\log_{10} W_\mu$  falls into a *low brightness region* in which  $p_\lambda$  is approximately constant and small, a *transitional region* in which  $p_\lambda$  increases with  $\log_{10} W_\mu$ , and a *high brightness region* in which  $p_\lambda$  is again approximately constant but has a high value. Turning to the curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$ , we note that

to begin with the gradient of curve I increases with  $\log_{10} W_\mu$  and approaches a constant value. Then at about the beginning of the transitional region the gradient decreases with increase in  $\log_{10} W_\mu$ . Subsequently it increases again and finally approaches a constant value. Curve II behaves similarly *except that the decrease in the gradient of the curve occurs at about the end of the transitional region*. Graphs of the logarithm of the parafoveal l.b.i. against the logarithm of the conditioning brightness exhibiting a "change of law" of this kind were first observed using white light for both test stimulus and conditioning stimulation and their interpretation in terms of the duplicity theory has already been given (Stiles and Crawford 1932, 1934). It is assumed that if we could determine the l.b.i.  $(U_\lambda)_s$  of the rod system acting alone we should obtain a curve of  $\log_{10}(U_\lambda)_s$  against  $\log_{10} W_\mu$  similar to curve A of fig. 17. The cone system acting alone would give, on the other hand, a curve of  $\log_{10}(U_\lambda)_p$  against  $\log_{10} W_\mu$  similar to curve B. In the practical case when both systems are in action, the measured  $\log_{10} U_\lambda$  would coincide with  $\log_{10}(U_\lambda)_s$  at low brightnesses where  $\log_{10}(U_\lambda)_s$  is considerably smaller than  $\log_{10}(U_\lambda)_p$ , and with  $\log_{10}(U_\lambda)_p$  at high brightnesses where the reverse is true. In the neighbourhood of the point of intersection of curves A and B, where  $\log_{10}(U_\lambda)_s$  and  $\log_{10}(U_\lambda)_p$  have comparable values the observed  $\log_{10} U_\lambda$  would be smaller than either so that the experimental curve would follow some such course as that indicated by the dotted line C. Curve C is made up of three sections. a rod section at low brightnesses where C is coincident with A, a cone section at high brightnesses where C is coincident with B and a mixed section at intermediate brightnesses where C lies below both the curves A and B.

Accepting the view that the change of law in curves I and II of fig. 15 is produced in the way just described, we obtain a simple explanation of curve III. Suppose in fig. 17, curves A and B refer to central entry of the test stimulus. Assume now that the rods show no directional sensitivity at any brightness of the central field. Curve A will then apply equally for peripheral and central entry.\* Suppose, on the other hand, that the cones show a *pronounced and constant* directional sensitivity at all brightness levels. Then the curve of  $\log_{10}(U_\lambda)_p$  against  $\log_{10} W_\mu$  for peripheral entry of the test stimulus (curve B') will have the same shape as curve B but will be

\* Since  $p_\lambda$  has a small positive value in the low brightness region of curve III (fig. 15), the rods probably show a slight directional effect when stimulated with  $\lambda = 580 \text{ m}\mu$ . For simplicity this effect has been ignored in constructing the curves of fig. 17 which are primarily intended to illustrate the principle of the suggested explanation.



FIG. 14

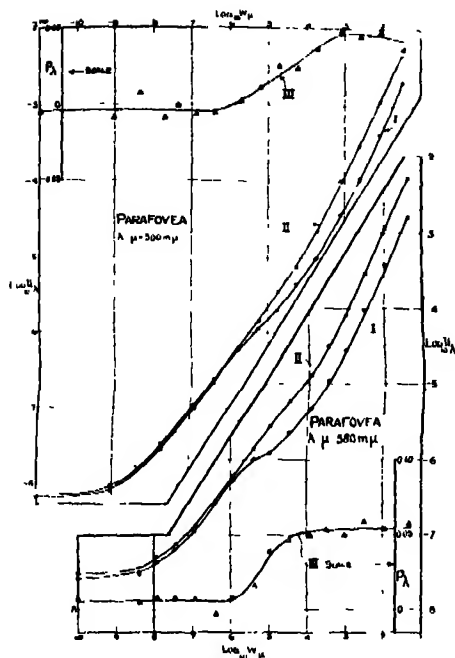


FIG. 15

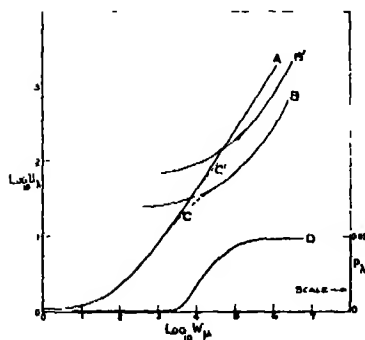


FIG. 17. Explanation of the results for parafoveal vision, in terms of the duplicity theory.

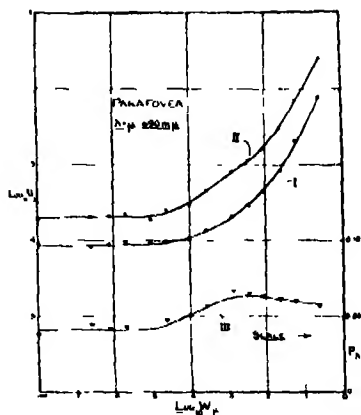


FIG. 16

FIGS. 14-16. For key see p. 81 of text.

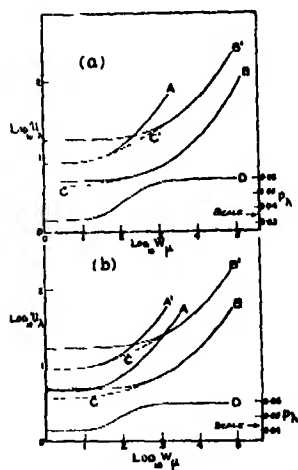


FIG. 18. Alternative explanations of the results for red light. In (b), A and A' refer to the rods and to central and peripheral entry respectively.

displaced parallel to the axis of  $\log_{10}(U_\lambda)$  to higher values. The resultant curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  when both rod and cone systems are in operation will be represented for peripheral entry by curve  $C'$ . By taking the difference of curves  $C'$  and  $C$  and applying formula (1) we obtain the variation of  $p_\lambda$  with  $\log_{10} W_\mu$  as curve  $D$ .

The main features of the experimental curves of fig. 15 are all reproduced in the corresponding curves  $C$ ,  $C'$  and  $D$  of fig. 17. For this case the duplicity theory, together with the assumption of constant directional sensitivities for the rod and cone systems, provides a possible explanation of the change in directional sensitivity with brightness level.

The experimental curves for  $\lambda = \mu = 500 \text{ m}\mu$  (fig. 14) are generally similar to those for  $\lambda = \mu = 580 \text{ m}\mu$  and may be similarly explained. There are differences, however. The transitional region in curve III extends over a wider range, and the change of law in the curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  is observable only for curve I. These differences may be due to the rod and cone curves  $A$  and  $B$  (fig. 17) being so displaced that they intersect at a smaller angle.

For  $\lambda = \mu = 690 \text{ m}\mu$  (fig. 16), we may apply the above explanation in the way shown schematically in fig. 18*a*. There is an alternative possibility however; the rods when stimulated with red light may exhibit a well-developed directional sensitivity, although a smaller one than the cones. The scheme of fig. 18*b* would then apply. We are faced here with the uncertainty to which reference was made at the end of the previous section.

The more general case when test stimulus and central field have different wave-lengths will now be examined. Figs. 19 and 20 give respectively the results of two-point determinations for the combinations  $\lambda = 580$ ,  $\mu = 500$  and  $\lambda = 450$ ,  $\mu = 520$ . With these combinations the transition from rod to cone vision in curves I and II and the corresponding change in the directional sensitivity measured by  $p_\lambda$  are very clearly shown. The results are in good accord with the explanation illustrated by the curves of fig. 17.

With the combination  $\lambda = 500$ ,  $\mu = 690$ , the results are of a different character (fig. 21). Despite the large scatter in the experimental values we may admit that on the whole there is no systematic variation of  $p_\lambda$  with brightness level. The curve relating  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  is nearly the same for central and peripheral entry and a "change of law" of the kind previously obtained is not in evidence. These facts suggest that at all values of  $\log_{10} W_\mu$ , the test stimulus is seen by rod vision. In our view this result is obtained because the rod curve (curve  $A$ , fig. 17) lies below and to the right of the two cone curves ( $B$  and  $B'$ , fig. 17) and does not intersect them. The results for the combination  $\lambda = 500$ ,  $\mu = 660$  (fig. 22) allow of a similar

FIG. 19

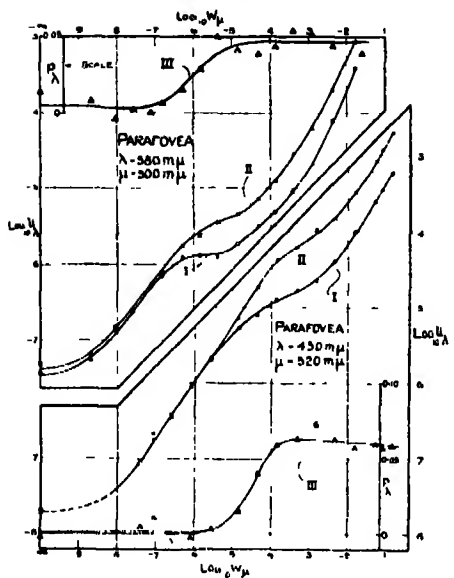


FIG. 20

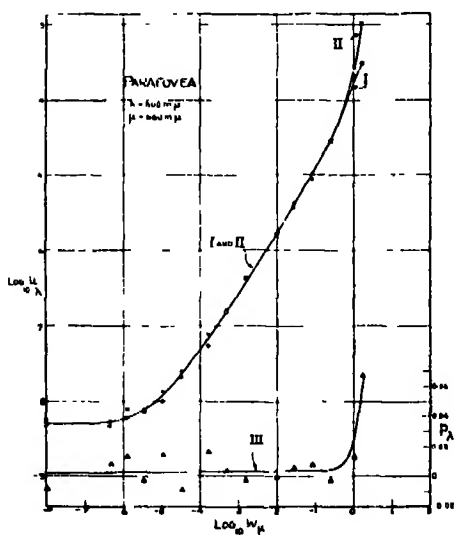


FIG. 22

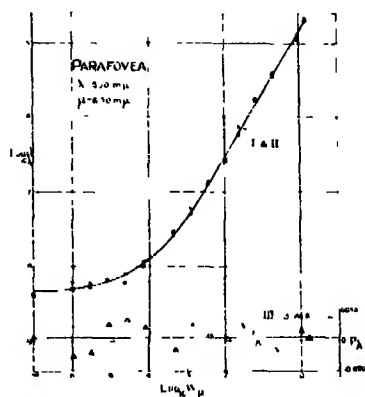


FIG. 21

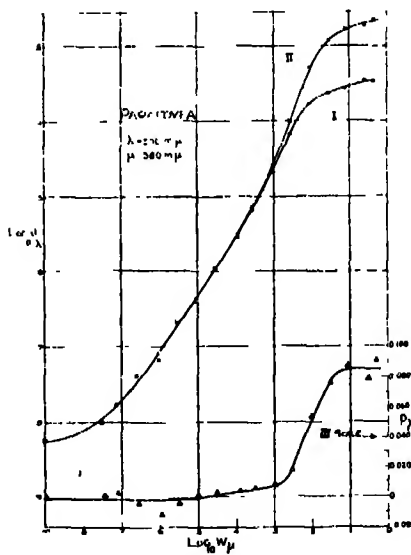


FIG. 23

FIGS. 19-23. Key as for figs. 14-16.

interpretation, except that at the highest brightness level  $p_\lambda$  rises sharply. This rise is believed to be genuine but its explanation depends on a new factor to be dealt with later.

The interpretation of the results may now be carried a stage further. For each combination of  $\lambda$  and  $\mu$ , we have envisaged a rod curve  $A$  and a cone curve  $B$ , but nothing has been said of the connection between the rod curves or the cone curves for different combinations. In the brightness range where curves I and II of fig. 22 are indistinguishable, the common curve according to our interpretation is purely a rod curve. A tracing of this curve can be fitted approximately over the rod sections of all the experimental curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu^*$  which possess an identifiable rod section. Thus to a first approximation the rod curve has the same shape for all combinations  $\lambda$  and  $\mu$  for which it can be observed and is merely displaced (without rotation) to different positions with respect to the axes. Moreover, for two combinations with the same  $\mu$  but different  $\lambda$ 's the rod curves are found to have approximately the same positions with respect to the axis of  $\log_{10} W_\mu$  but are relatively displaced in a direction parallel to the axis of  $\log_{10} U_\lambda$ . On the other hand, for two curves with the same  $\lambda$  but different  $\mu$ 's the rod curves have approximately the same positions with respect to the axis of  $\log_{10} U_\lambda$  but are relatively displaced parallel to the axis of  $\log_{10} W_\mu$ .

It follows from these experimental conclusions that the relation between  $\log_{10}(U_\lambda)_s$   $\{(U_\lambda)_s$  stands for the l.b.i. of the rod system acting alone} and  $\log_{10} W_\mu$  must be of the form

$$\log_{10}(U_\lambda)_s + \log_{10} s_\lambda = F\{\overline{\log_{10} W_\mu} + \overline{\log_{10} S_\mu}\}, \quad (2)$$

where  $s_\lambda$  depends only on  $\lambda$ ,  $S_\mu$  depends only on  $\mu$  and  $F(x)$  is a fixed function of  $x$  determined by the shape of the rod curve. Relation (2) may be written

$$\log_{10} \overline{s_\lambda(U_\lambda)_s} = F\{\log_{10} \overline{W_\mu S_\mu}\}.$$

This implies that  $s_\lambda(U_\lambda)_s$  is a fixed function of  $S_\mu W_\mu$ . It is equally true that  $1/\overline{s_\lambda(U_\lambda)_s}$  is a fixed function of  $S_\mu W_\mu$ , which we may call  $\xi_s\{\overline{W_\mu S_\mu}\}$ .

Thus

$$1/\overline{(U_\lambda)_s} s_\lambda = \xi_s\{\overline{W_\mu S_\mu}\},$$

or

$$1/(U_\lambda)_s = s_\lambda \xi_s\{\overline{W_\mu S_\mu}\}. \quad (3)$$

The experimental data determine only the relative values of  $s_\lambda$  at different wave-lengths  $\lambda$ , and the relative values of  $S_\mu$  at different wave-lengths  $\mu$ .

\* Including curves for a number of combinations for which the results cannot be given in full.

It is convenient to remove these ambiguities, (a) by giving the fixed function  $\xi_s(x)$  the value unity when  $x = 0$  so that  $s_\lambda$  is the reciprocal of the l.b.i. of the rods when  $W_\mu$ , the intensity of the central field, is zero and (b) by giving the fixed function  $\xi_s(x)$  the value 0.1 when  $x = 1$ . It follows from condition (b) that if  $W_\mu S_\mu = 1$ ,  $\xi_s(W_\mu S_\mu) = 0.1$  and  $1/(U_\lambda)_s$  has one-tenth its value for a central field of zero intensity. Thus  $S_\mu$  equals the reciprocal of the intensity of the central field necessary to reduce  $1/(U_\lambda)_s$  to one-tenth, or to increase  $(U_\lambda)_s$  to ten times its value for zero field. In this sense  $S_\mu$  measures the sensitivity of the rod system to the "conditioning" effect of central fields of different wave-lengths.

It is easily deduced from equation (3) that the curve of  $(-\log_{10} \xi_s(x))$  against  $\log_{10} x$  must have the same shape as the rod section of any experimental curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$ . The plot of  $(-\log_{10} \xi_s(x))$  against  $\log_{10} x$  shown as curve 1 in fig. 36 is simply the rod section of curves I and II of fig. 22 placed in such a position with respect to the axes that the two conditions  $\xi_s(x) = 1$  when  $x = 0$  and  $\xi_s(x) = 0.1$  when  $x = 1$  are satisfied. Thus curve 1 of fig. 36 completely defines the fixed function of the rods,  $\xi_s(x)$ .

The curve of  $\log_{10} s_\lambda$  against  $\lambda$  will coincide approximately with the parafoveal curve of  $\log_{10}(1/U_\lambda)$  against  $\lambda$  for zero field (fig. 11), except in the red end of the spectrum where  $\log_{10} s_\lambda$  will have slightly lower values owing to the participation of the cones in determining  $U_\lambda$ . The mean values of  $\log_{10} S_\mu$  obtained from the rod sections of the various experimental curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  are given in Table II. The variation of  $\log_{10} S_\mu$  with  $\mu$  is very similar to the variation of  $\log_{10} s_\lambda$  with  $\lambda$ , as is shown by the approximate constancy of  $\overline{\log_{10} s_\lambda} - \overline{\log_{10} S_\mu}$ , ( $\lambda = \mu$ ).

TABLE II

$\mu$	...	440	500	520	580	660	690
$\log_{10} S_\mu$		6.6	7.3	7.1	6.4	4.1	3.1
$\overline{\log_{10} s_\lambda} - \overline{\log_{10} S_\mu}$ at $\lambda = \mu$		1.0	0.9	1.1	0.9	1.0	1.1

This means that to a first approximation the relative values of lights of different wave-length in producing a threshold stimulation of the rods and in bringing the rods to a given level of stimulation are the same.

The above discussion applies in the first instance to the response of the rods to test stimuli entering centrally. To allow for entry at other points, the factor  $10^{-\nu \lambda s^{(d)} d_m^2}$  may be introduced in the expression for  $1/(U_\lambda)_s$  which now becomes

$$1/(U_\lambda)_s = 10^{-\nu \lambda s^{(d)} d_m^2} s_\lambda \xi_s(W_\mu S_\mu), \quad (4)$$

where  $p_{\lambda s}$  determines the directional effect of the rods. For  $\lambda$  not greater than  $600 \text{ m}\mu$  we may identify  $p_{\lambda s}$  with the  $p_\lambda$  plotted in fig. 7. For longer wave-lengths  $p_{\lambda s}$  may be less than  $p_\lambda$ .

Turning now to the cones, we consider the possibility that the l.b.i. of the cones  $(U_\lambda)_p$  can be represented by an expression similar in form to that already obtained for the rods. For the combination  $\lambda = 580$ ,  $\mu = 500$  (fig. 19), the cone section of curve I extends from the highest brightness levels down to a brightness  $(\log_{10} W_\mu = \bar{6}\cdot2 \text{ approx.})$  where the curve becomes nearly horizontal and the l.b.i. of the cones has become constant. We may take a tracing of this pure cone curve and try to fit it over the cone sections of the curves for other combinations  $\lambda$ ,  $\mu$ , which have an identifiable cone section. The process is much less satisfactory than in the case of the rods, partly because for most of the available curves the mixed section of the curve where both rods and cones are operative intervenes before the flat part of the cone curve is reached.

A more radical difficulty is revealed by the measurements for the combination  $\lambda = 500$ ,  $\mu = 580$  (fig. 23). The curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  show a sharp "change of law" when  $\log_{10} W_\mu$  is in the neighbourhood of  $\bar{2}$  and at about the same brightness  $p_\lambda$  rises sharply. As before we attribute these features to a transition from rod to cone vision of the test stimulus. Comparing curves I and II of fig. 23 with the corresponding curves of fig. 15 for which  $\mu$  has the same value ( $580 \text{ m}\mu$ ), it is easy to see that the cone sections for the two cases cannot arise from a curve of fixed shape which is merely displaced parallel to the axis of  $\log_{10} U_\lambda$  by varying amounts depending on the value of  $\lambda$ . If this were so the gradients of the cone sections in figs. 23 and 15 at a given value of  $\log_{10} W_\mu$  would be the same, for these gradients are unaffected by displacements parallel to the axis of  $\log_{10} U_\lambda$ . Actually at  $\log_{10} W_\mu = \bar{2}\cdot6$ , the gradient (tangent) of curve I equals  $0\cdot2$  in fig. 23 and  $1\cdot0$  in fig. 15. Similarly, if we compare the cone sections of fig. 23 with those of fig. 14 for which  $\lambda$  has the same value, it is clear that the cone sections for the two cases cannot arise from a curve of fixed shape which is merely displaced parallel to the axis of  $\log_{10} W_\mu$  by varying amounts depending on the value of  $\mu$ .

It must be concluded from the above that the cone curves for different combinations  $\lambda$ ,  $\mu$  cannot be represented by an expression similar to (4). Other anomalies in the cone curves confirm this conclusion. Thus the simple idea of a curve of fixed shape which is displaced parallel to the axes of  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  by amounts depending on the values of  $\lambda$  and  $\mu$  respectively, breaks down for cone vision although it holds for rod vision. The more complex behaviour of the parafoveal cones is elucidated by the

measurements for foveal vision of the stimulus which give the response of a cone system unobscured by the presence of a rod response. It appears that to explain the response of the cones the duplicity theory must be supplemented with the idea of three distinctive types of cone, an idea which is, of course, inherent in many forms of the trichromatic theory.

#### 8. CENTRAL FIELD ILLUMINATED: FOVEA

When the eye is adapted to a field of zero brightness, the fovea exhibits a pronounced directional sensitivity for all wave-lengths of the test stimulus. Measurements made with the central field illuminated show that this pronounced directional sensitivity is retained whatever the intensity and wave-length of the central field. These measurements consisted in the main of two-point determinations in which  $\lambda$  and  $\mu$  were kept constant while the energy intensity of the central field  $W_\mu$  was increased in steps from zero upwards. The determination of  $\log_{10} U_\lambda$  for "central" entry of the test stimulus was made at  $d_o = +\frac{3}{4}$  or  $+1$  mm. (temporal) as it was known that for the fovea of this eye  $d_m$  equalled about 0.9 mm. The determination for peripheral entry was made, in most cases, at  $d_p = -3$  mm. (nasal). Except where the contrary is indicated, the rays forming the central field entered the eye through the centre of the pupil, i.e. at  $d = 0$ . An adequate period of dark-adaptation preceded the run. A few minutes' adaptation to the higher brightness levels was necessary before steady values could be obtained.

The results obtained when the test stimulus and central field had the same wave-length are plotted in figs. 24-27. For  $\lambda = \mu = 580$  m $\mu$ ,  $p_\lambda$  remains practically constant at all brightness levels. For the other wave-lengths there is some variation in  $p_\lambda$  which can be followed despite the scatter of the experimental points. The curves relating  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  for wave-lengths 500, 580 and 690 m $\mu$  are of nearly the same shape, that is to say, they can be obtained by moving a single curve without rotation to different positions with respect to the axes. The curves for the wave-length 430 m $\mu$ , however, show a significant difference in shape from the others. Thus even in the restricted case when test stimulus and central field have the same wave-length, the notion of a curve of fixed shape which is merely displaced to different positions with respect to the axes breaks down.

Figs. 28-35 give the results for eight combinations in which the wave-length of the test stimulus lies in the range 430 to 500 m $\mu$  while  $\mu$ , the wave-length of the central field, is considerably greater than  $\lambda$  and lies in

the range 500 to 590  $m\mu$ . For every combination except  $\lambda = 430$ ,  $\mu = 500$ , the curves of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  show a "change of law" of the kind already met with for parafoveal vision, and  $p_\lambda$  shows a corresponding increase from an approximately constant value in a range of low brightnesses to an approximately constant but different value in a range of high brightnesses.

These features of the results can be explained by assuming that there are two types of cone in operation, each with distinctive properties. In fig. 31a\* (p. 95), for example, we assume that the left-hand section of curve I, extrapolated as a broken line to higher values of  $\log_{10} W_\mu$ , is the curve connecting  $\log_{10} U_\lambda$  with  $\log_{10} W_\mu$  which would be obtained if cones of one type (type *X*) were acting alone. Similarly we assume that the right-hand section of curve I, extrapolated as a dotted line, is the curve which would be obtained if cones of the other type (type *B*) were acting alone. These component curves will lead to the observed curve when both types are in action if the observed l.b.i. coincides approximately with the smaller of the l.b.i.'s of the two types except where these have equal or nearly equal values.

Curve II is obtained by displacing the two component curves of curve I without rotation in a direction parallel to the axis of  $\log_{10} U_\lambda$ . The component curve of type *B* must be displaced by a greater amount than that of type *X*. This connexion between curves I and II is explained if the directional sensitivity of each type of cone is independent of brightness level and type *B* has a greater directional sensitivity than type *X*.

A similar explanation may be given of the results for the other combinations in this group if we admit that as  $\lambda$  decreases the vertical separation of the *X* and *B* component curves diminishes until for  $\lambda = 430 m\mu$  (fig. 35) the *X* curve lies above the *B* curve at all values of  $\log_{10} W_\mu$  and plays little if any part in determining the resultant curve. For  $\lambda = 490$ ,  $\mu = 590$  (fig. 29), curves I and II show a new feature. The *B* component curves become suddenly horizontal at  $\log_{10} W_\mu = 1.1$  approx. There is an indication of a similar effect for the combination  $\lambda = 500$ ,  $\mu = 580$  (fig. 28). This effect might be attributed to a third type of cone but as shown below such an explanation is unlikely. It appears instead that for certain wave-lengths  $\mu$ , the conditioning effect of the central field on the *B* cones is limited, that is to say, however great the intensity of the central field it cannot increase the l.b.i. of the *B* cones above a certain value.

Setting aside the anomaly just noted, it is found that those sections of the experimental curves which are identified with the *X* or *B* component curves can all be fitted approximately with a curve of fixed shape (curve 2

\* The experimental data in this figure are the same as in fig. 31.



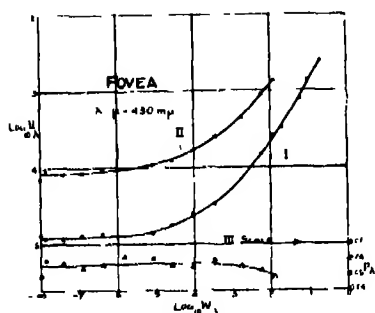


FIG. 24

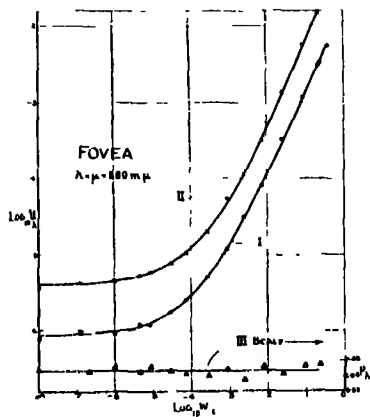


FIG. 26

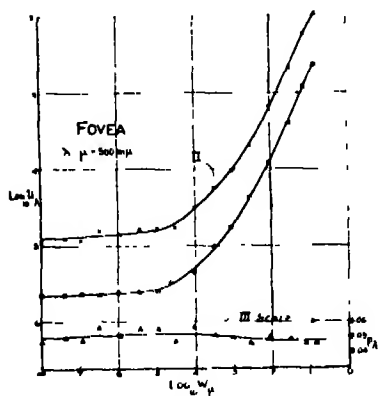


FIG. 25

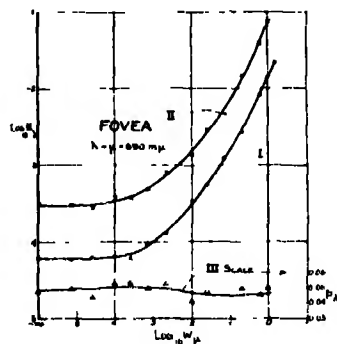


FIG. 27

FIGS. 24-27. Key as for figs. 14-16.

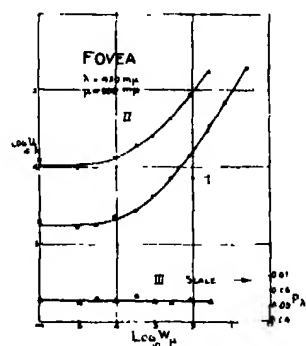
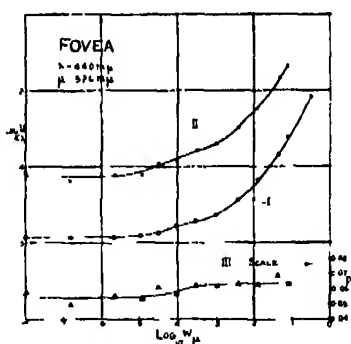
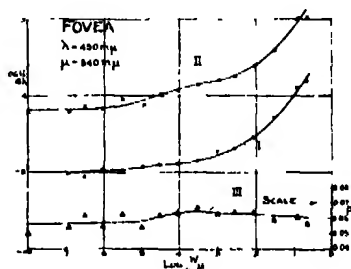
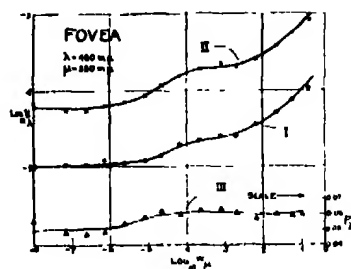
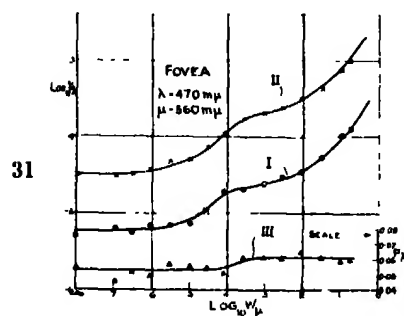
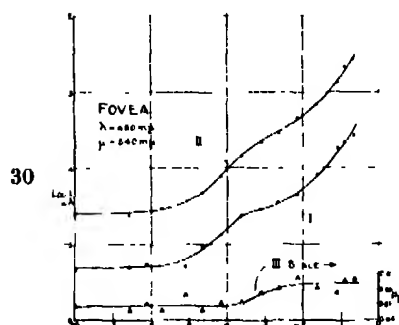
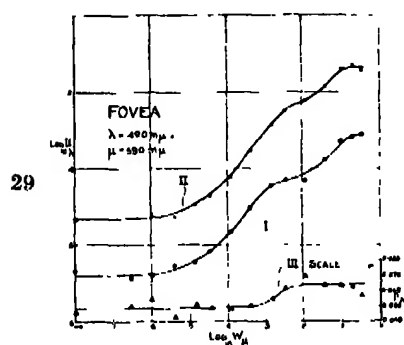
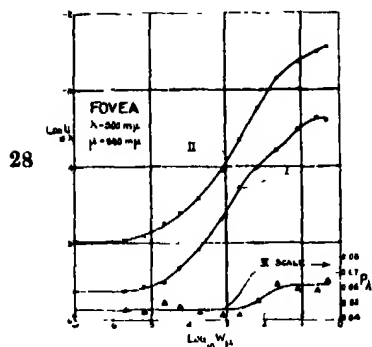
of fig. 36) which is moved (without rotation) to different positions with respect to the axes.

A series of twenty-one runs was then made with a test stimulus of wave-length  $480\text{ m}\mu$  and with central fields of wave-lengths ranging from  $430$  to  $700\text{ m}\mu$ . The l.b.i. was determined only for central entry of the test stimulus. The rays forming the central field entered the eye through the same point as the rays forming the test stimulus. Some of the results obtained are plotted in fig. 37. The vertical scale of fig. 37 is correct for all the data, but the horizontal scale is correct only for  $\mu = 700\text{ m}\mu$ . For other values of  $\mu$  the origin of the horizontal scale has been shifted by arbitrary amounts to give a convenient spacing of the curves. For all wave-lengths of the central field except  $430$  and  $480\text{ m}\mu$ , the experimental results can be represented approximately by the two curves shown, which are reproductions of curve 2 of fig. 36. Moreover, the upper of the two curves, which is associated with the *B* cones, flattens out at approximately the same value of  $\log_{10} U_{\lambda}$  ( $\bar{5}\cdot5$ ) for all values of  $\mu$ . The lower curves, which are associated with the *X* cones, also flatten out at a common value, about  $\bar{6}\cdot8$  in this case. This is to be expected, however, since with a central field of zero intensity ( $\log_{10} W_{\mu} = -\infty$ ),  $\log_{10} U_{\lambda}$  must be the same whatever the value of  $\mu$ .<sup>\*</sup> The horizontal separation of the *B* and *X* curves decreases as  $\mu$  decreases below about  $560\text{ m}\mu$  until at  $\mu = 480\text{ m}\mu$  the two curves no longer intersect and the results are represented by the *X* curve only. The limited conditioning of the *B* cones at high brightnesses is in evidence for  $\mu = 580$  and  $610\text{ m}\mu$ , but not for  $\mu = 560\text{ m}\mu$  or less. It is not observable for  $\mu = 640$  or  $700\text{ m}\mu$ , but this may be because the brightness of the central field could not be made sufficiently high for these wave-lengths.

A selection from a similar set of nineteen runs with  $\lambda = 430\text{ m}\mu$  and with  $\mu$  varying from  $640$  to  $410\text{ m}\mu$  is shown in fig. 38. For  $\mu$  greater than  $460\text{ m}\mu$  a single curve of the form of curve 2, fig. 36, can be drawn to represent the results apart from the limited conditioning effect which occurs for  $\mu = 580$  and  $620\text{ m}\mu$ . For  $\mu$  equal to  $460\text{ m}\mu$  or less two curves of the standard shape are required as shown. The two curves intersect before the upper curve has flattened out, but since the shape of the curves is fixed it can still be deduced that the upper curve would flatten out to about the same value of  $\log_{10} \mu_{\lambda}$  ( $\bar{5}\cdot5$ ) in each case.

In a third series of eleven runs, the wave-length of the central field was equal to  $600\text{ m}\mu$  in every case and the wave-length of the test stimulus was varied from  $410$  to  $510\text{ m}\mu$  (fig. 39). In the figure, the horizontal scale is correct for all combinations  $\lambda, \mu$ . The vertical scale is correct only for

\* Apart from day-to-day variations in the sensitivity of the eye.



FIGS. 28-35. Key as for figs. 14-16.

$\lambda = 510$ ,  $\mu = 600$  and is displaced by arbitrary amounts for the other cases. For  $\lambda = 510$ ,  $\mu = 600$ , the upper and lower curves are associated with the  $B$  and  $X$  cones respectively. As  $\lambda$  decreases, the component curves maintain their positions with respect to the horizontal axis but they are displaced by different amounts parallel to the vertical axis. For  $\lambda = 410$  and  $430 \text{ m}\mu$ , the results are represented approximately by the  $B$  curve only. In every case the  $B$  curve becomes abruptly horizontal at  $\log_{10} W_\mu = 1.2$  approx. Thus, whatever the wave-length of the test stimulus, a central field of wave-length  $600 \text{ m}\mu$  has a limited conditioning effect on the  $B$  cones and can raise the l.b.i. of the  $B$  cones to about 4.3 times the value for zero field but no more. Taking account of all the data obtained, it appears probable that the same thing is true for a central field of any wave-length exceeding  $570 \text{ m}\mu$  although insufficient intensity of the central field prevents our observing the effect for  $\mu = 640 \text{ m}\mu$  or more.

The above results show that the l.b.i. of the  $B$  cones acting alone  $(U_\lambda)_b$  can be represented approximately by the following expression, which is similar to that used for the rods,

$$1/(U_\lambda)_b = b_\lambda \xi\{\bar{W}_\mu \bar{B}_\mu\} 10^{-\nu_{\lambda b}(d-d_m)^2}, \quad (5)$$

where  $b_\lambda$  is independent of  $\mu$  and equals the reciprocal of the l.b.i. of the  $B$  cones when the central field has zero energy,

$\bar{B}_\mu$  is independent of  $\lambda$  and equals the reciprocal of the energy of the central field necessary to raise the l.b.i. of the  $B$  cones to ten times the value for zero field,

$\xi(x)$  is a fixed function of  $x$ . Curve 2 of fig. 36 gives a plot of  $\{-\log_{10} \xi(x)\}$  against  $\log_{10} x$ ,

$p_{\lambda b}$  determines the directional sensitivity of the  $B$  cones for a test stimulus of wave-length  $\lambda$ .

For wave-lengths of the central field greater than  $570 \text{ m}\mu$ , the modified form of  $\xi(x)$  shown as curve 3 in fig. 36 must be used to allow for the limited conditioning effect obtained with these wave-lengths. In such cases  $\bar{B}_\mu$  is defined as "the reciprocal of the energy of the central field which would raise the l.b.i. of the  $B$  cones to ten times the value for zero field if the limited conditioning effect did not operate".

We may adopt an analogous expression for the l.b.i. of the  $X$  cones acting alone,  $(U_\lambda)_x$ . We put

$$1/(U_\lambda)_x = x_\lambda \xi\{\bar{W}_\mu \bar{X}_\mu\} 10^{-\nu_{\lambda x}(d-d_m)^2}, \quad (6)$$

where  $x_\lambda$ ,  $\bar{X}_\mu$ ,  $p_{\lambda x}$  are defined in precisely the same way as  $b_\lambda$ ,  $\bar{B}_\mu$  and  $p_{\lambda b}$

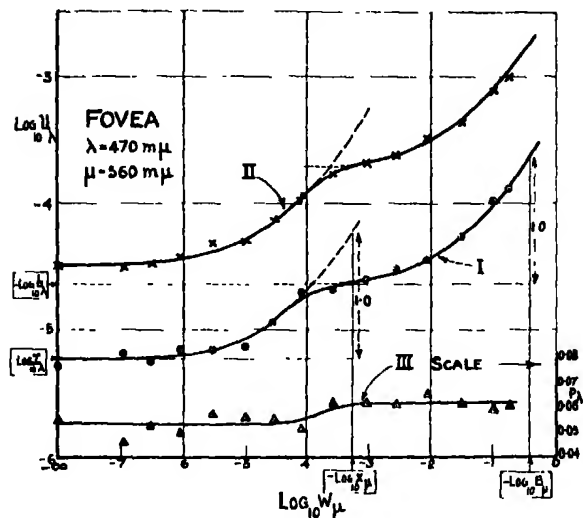


FIG. 31a

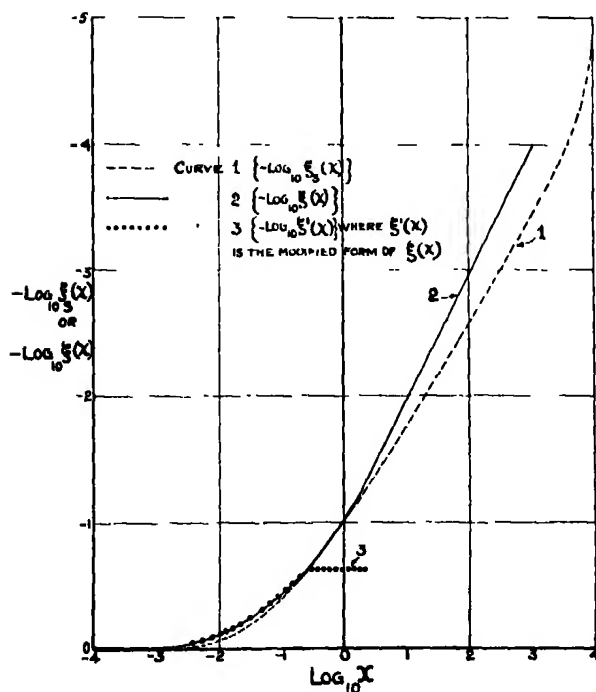


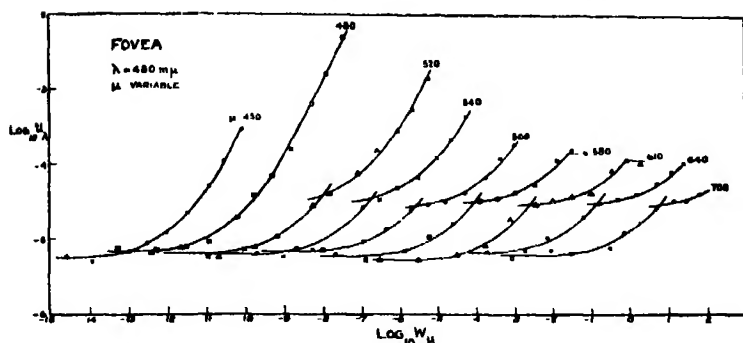
FIG. 36. The rod function  $\xi_r(x)$ , the cone function  $\xi(x)$ , and the modified cone function  $\xi'(x)$  applicable to the blue cones when  $\mu$  exceeds  $570 \text{ m}\mu$ .

respectively.  $\xi(x)$  is, to a sufficient approximation, the same function as for the  $B$  cones. No limited conditioning effect has been observed for the  $X$  cones.

From a curve such as curve 1 of fig. 31*a* where the  $X$  and  $B$  components are well defined the values of  $b_\lambda$ ,  $B_\mu$ ,  $x_\lambda$  and  $X_\mu$  are easily obtained in the way illustrated in the figure. For other cases the process is less certain. The mean results of all the determinations of  $b_\lambda$ ,  $x_\lambda$ ,  $B_\mu$  and  $X_\mu$  (for  $\lambda$  less than 520  $m\mu$ ) are plotted in figs. 40 and 41. The curve of  $\log_{10} b_\lambda$  against  $\lambda$  (curve 1, fig. 40) has a maximum at  $\lambda = 440$   $m\mu$  approximately, which means that the  $B$  cones, or the blue cones as we may now call them, are most sensitive to test stimuli of wave-length 440  $m\mu$ . Owing to the limited conditioning effect the l.b.i. of the blue cones has a maximum value  $M_\lambda$  for central fields of wave-length  $\mu$  greater than 570  $m\mu$ . Curve 2 of fig. 40 shows the observed variation of  $\log_{10} 1/M_\lambda$  with  $\lambda$ . Curve 2 is obtainable from curve 1 by a simple vertical displacement. Thus, if what we have called the limited conditioning effect were due to a third type of cone, the relative spectral sensitivity to test stimuli of different wave-length would be the same as for the blue cones.

The curve of  $\log_{10} B_\mu$  against  $\mu$  (curve 1, fig. 41), shows that the conditioning effect of the central field on the blue cones is greatest for  $\mu = 445$   $m\mu$  approx. The sensitivity curves of the blue cones to test stimuli of different wave-length (curve 1, fig. 40) and to central fields of different wave-length (curve 1, fig. 41) appear to be slightly different in shape in the region of the spectrum (410–510  $m\mu$ ) where both can be followed although the difference may be experimental error. As  $\mu$  increases beyond 510  $m\mu$  the curve of  $\log_{10} B_\mu$  descends steadily until at  $\mu = 570$   $m\mu$  it swings round and becomes horizontal to descend again steadily for  $\mu$  greater than 600  $m\mu$ . This change of form at  $\mu = 570$   $m\mu$  may be connected with the limited conditioning effect of central fields for which  $\mu$  exceeds 570  $m\mu$ . If the limiting conditioning effect were due to a third type of cone, the appropriate sensitivity curve to central fields of different wave-length would have to exhibit something like a discontinuity in the neighbourhood of  $\mu = 570$   $m\mu$ . The simpler view and the one adopted here is that we are dealing with a special property of the blue cones.

We are naturally led to consider whether two types of cone are sufficient to explain all measurements of the present type. Fig. 39 shows that as  $\lambda$  increases the vertical separation of the  $B$  and  $X$  component curves increases until at  $\lambda = 510$   $m\mu$  the  $B$  component is responsible only for the upper extremity of the experimental curve. We may anticipate that for still greater values of  $\lambda$  (540  $m\mu$  or more) the process will be continued and the



experimental curve will consist simply of the  $X$  component.\* Measurements show, however, that with test stimuli of wave-length greater than  $540\text{ m}\mu$  the relation between  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  cannot be represented by the standard curve (curve 2, fig. 36) for all wave-lengths of the central field. In some cases, such as that shown in fig. 42, a change of law of the familiar kind is in evidence. Thus the  $X$  cone system is not a simple system. One possibility is that two cone systems, which we may call the green and red systems, are in operation, whose l.b.i.'s are represented by the expressions:

$$1/(U_\lambda)_g = g_\lambda \xi\{W_\mu G_\mu\} 10^{-p_{\lambda g}(d-d_m)^2}, \quad (7)$$

$$1/(U_\lambda)_r = r_\lambda \xi\{W_\mu R_\mu\} 10^{-p_{\lambda r}(d-d_m)^2}, \quad (8)$$

where  $g_\lambda$ ,  $G_\mu$ ,  $p_{\lambda g}$  and  $r_\lambda$ ,  $R_\mu$  and  $p_{\lambda r}$  have similar definitions to those given for  $b_\lambda$ ,  $B_\mu$  and  $p_{\lambda b}$ . The above expressions would then take the place of the single expression (6) for the  $X$  cones. Many more measurements will be required for the full working out of this view since it appears that the spectral sensitivities of the red and green systems do not differ so radically as do those of the blue and  $X$  systems. From the results so far obtained it is probable that for test stimuli of wave-length not greater than  $510\text{ m}\mu$ , the  $X$  component curve differs little from the curve of one of the two systems introduced above. Thus the values of  $\log_{10} x_\lambda$  and  $\log_{10} X_\mu$  plotted in figs. 40 and 41 may be identified with  $\log_{10} g_\lambda$  and  $\log_{10} G_\mu$  respectively. Using the data of fig. 42 and other similar data a first attempt can be made to construct the sensitivity curves of the green and red cones. The curves so obtained are shown as broken lines in figs. 40 and 41. In fig. 41, the three lower curves give the values obtained for  $p_{\lambda b}$ ,  $p_{\lambda g}$  and  $p_{\lambda r}$  which measure the directional sensitivities of the blue, green and red cones. The difference between  $p_{\lambda b}$  and  $p_{\lambda g}$  for  $\lambda$  less than  $500\text{ m}\mu$  is well established. The crossing of the curves for  $p_{\lambda g}$  and  $p_{\lambda r}$  at  $\lambda = 620\text{ m}\mu$  is less certain. In figs. 40 and 41 all curves or parts of curves shown by broken lines must be regarded as tentative only.

A striking difference in properties between the blue cones and the red or green cones is indicated if we compare the sensitivity to a test stimulus of given wave-length with the sensitivity to the conditioning effect of a central field of the same wave-length. This comparison may be made by taking the differences  $\log_{10} b_\lambda - \log_{10} B_\mu$ ,  $\log_{10} g_\lambda - \log_{10} G_\mu$  and  $\log_{10} r_\lambda - \log_{10} R_\mu$  for  $\lambda = \mu$ .  $\log_{10} b_\lambda - \log_{10} B_\mu$  is of the order of 2.0 whereas  $\log_{10} g_\lambda - \log_{10} G_\mu$

\* This conclusion assumes that the sensitivity curves of the  $B$  and  $X$  components to test stimuli of different wave-length (curves 1 and 3 of fig. 40) will have approximately the same shape as the corresponding sensitivity curves to central fields of different wave-length (curves 1 and 2 of fig. 41) which can be followed throughout the spectrum.



and  $\log_{10} r_\lambda - \log_{10} R_\mu$  equal about 2.7 and 2.8 respectively. The meaning of this result is apparent if we note that the fixed function  $\xi(x)$  approximates to  $0.11/x$  for sufficiently high values of  $x$ . Thus for central fields of sufficiently high brightness and for central entry of the test stimulus:

$$1/(U_\lambda)_b = b_\lambda \xi(\overline{W_\mu B_\mu}) = b_\lambda 0.11/W_\mu B_\mu$$

or 
$$\frac{(U_\lambda)_b}{W_\mu} = \frac{B_\mu}{0.11 b_\lambda}. \quad (9)$$

Now when  $\lambda = \mu$ ,  $(U_\lambda)_b/W_\mu$  represents in a certain sense the Fechner fraction of the blue cones acting alone. Similarly,  $(U_\lambda)_g/W_\mu = G_\mu/0.11 g_\lambda$  and  $(U_\lambda)_r/W_\mu = R_\mu/0.11 r_\lambda$  represent respectively the Fechner fractions of the green and red systems acting alone. Thus the fact that  $\log_{10} \overline{B_\mu} - \log_{10} \overline{b_\lambda}$  or  $\log_{10} B_\mu/b_\lambda$  exceeds  $\log_{10} \overline{G_\mu} - \log_{10} \overline{g_\lambda}$  or  $\log_{10} G_\mu/g_\lambda$  by approximately 0.7 means that the *limiting Fechner fraction* of the blue cones at high brightnesses is about five times that of the green cones.

Reverting for a moment to the rods, Table II shows that for  $\lambda = \mu$   $\log_{10} s_\lambda - \log_{10} S_\mu$  equals about 1.0. Comparison with the cones is complicated by the difference in shape of the rod and cone functions (curves 1 and 2 of fig. 3b). It can be deduced, however, that the Fechner fraction of the rods at high adapting brightnesses lies between 3 and 10 times the limiting Fechner fraction of the blue cones.

A brief reference must be made to the changes in the apparent colour of the test stimulus, when its intensity is just above the threshold, which are observed as the brightness of the central field is increased. In cases similar to that of fig. 31, there occurs a change in colour which corresponds broadly to the change in law of the experimental curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$ . In other cases where test stimulus and central field have the same wavelength, the test stimulus appears as a white flash.

#### 9. CHANGE IN THE POINT OF ENTRY OF THE RAYS FORMING THE CENTRAL FIELD. FOVEAL VISION

When the rays forming the central field are sent into the eye near the periphery of the pupil instead of at or near the centre, measurements of the l.b.i. for different points of entry of the test stimulus give a curve of  $\log_{10} U_\lambda$  against  $d$  of the same general shape as before. The main difference is that the absolute values of  $\log_{10} U_\lambda$  are in general smaller for peripheral than for central entry of a central field of fixed energy. This means that the

effectiveness of the central field in raising the l.b.i. above the value for zero field depends on the direction of incidence on the retina of the rays forming the central field. The magnitude of the directional effect for the central field is of the same order as that for the test stimulus. It is possible, however, that the curve of  $\log_{10} U_\lambda$  against  $d$  may not be of quite the same shape for different points of entry of the central field. A difference was observed in one set of measurements, but has not yet been confirmed.

The two sets of data of fig. 43 show the variation of  $\log_{10} U_\lambda$  with  $\log_{10} W_\mu$  ( $\lambda = 480$ ,  $\mu = 580$ ) for a test stimulus entering at  $d = +1$  (temp.) and for a central field entering at  $+1$  (temp.) (circle points) or  $-2\frac{3}{4}$  (nasal) (cross-points). To a first approximation each curve can be resolved into two component curves, corresponding to the blue and the green cones respectively, and the effect of changing the point of entry of the central field appears as a displacement of each component curve parallel to the axis of  $\log_{10} W_\mu$ . Similar results were obtained for the combination ( $\lambda = 480$ ,  $\mu = 600$ ). The horizontal displacement of each component curve determines the directional sensitivity of the corresponding type of cone to the conditioning effect of the central field just as the vertical separation of each component curve in fig. 31 determines the directional sensitivity to the test stimulus. It should be possible by this method to measure the directional sensitivity of the blue, green and red cones to the conditioning stimulation over the same range of values of  $\mu$  for which  $B_\mu$ ,  $G_\mu$  and  $R_\mu$  can be determined (see fig. 41).

When the point of entry of the central field is allowed to vary the expressions given above for  $1/(U_\lambda)_b$ ,  $1/(U_\lambda)_g$  and  $1/(U_\lambda)_r$  must be modified. As a tentative suggestion we may put

$$1/(U_\lambda)_b = b_\lambda 10^{-\nu \lambda b(d-d_m)^2} \xi \{W_\mu B_\mu 10^{-P_{\mu b}(d'-d_m)^2}\}, \quad (10)$$

and similar expressions for  $1/(U_\lambda)_g$  and  $1/(U_\lambda)_r$ , where  $d'$  is the point of entry of the central field and  $P_{\mu b}$  determines the directional sensitivity of the blue cones to a conditioning stimulation of wave-length  $\mu$ . This form assumes that the curve of  $\log_{10} U_\lambda$  against  $d$  has the same shape for all points of entry of the central field.

## 10. GENERAL DISCUSSION

Fig. 44 summarizes in a single diagram the connexion between the sensitivity and the condition of stimulation of the foveal retina to which we are led by the present work. We imagine that the eye views the  $10^\circ$  patch of

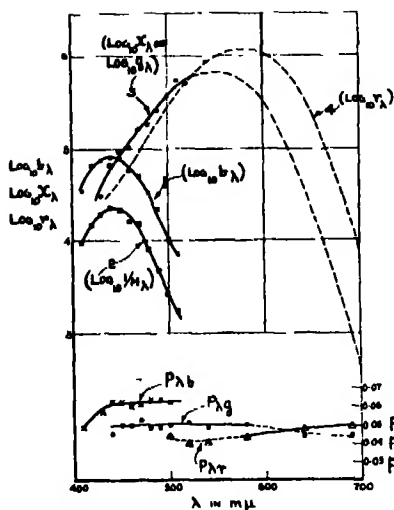


FIG. 40. Variation with  $\lambda$  of  $\log_{10} b_\lambda$ ,  $\log_{10} x_\lambda$  (or  $\log_{10} g_\lambda$ ),  $\log_{10} r_\lambda$ ,  $\log_{10} (1/M_\lambda)$ ,  $p_{\lambda b}$ ,  $p_{\lambda g}$  and  $p_{\lambda r}$ .

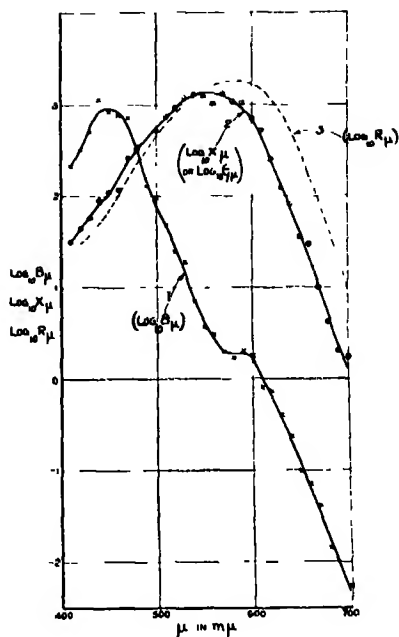


FIG. 41

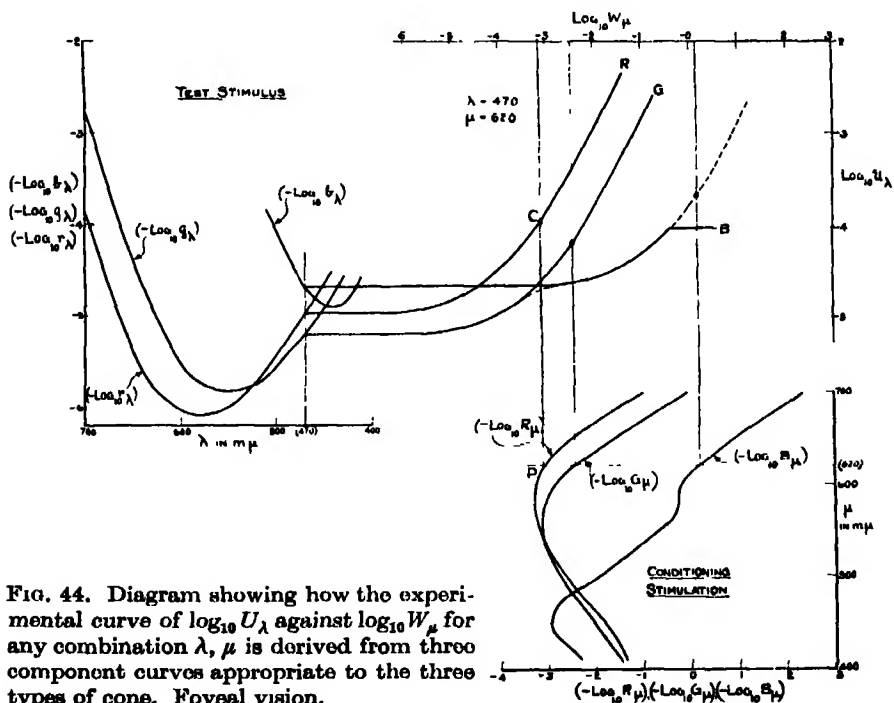


FIG. 44. Diagram showing how the experimental curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  for any combination  $\lambda, \mu$  is derived from three component curves appropriate to the three types of cone. Foveal vision.

light of wave-length  $\mu$  and energy  $W_\mu$  and that we determine the l.b.i.  $U_\lambda$ , for a monochromatic test stimulus of wave-length  $\lambda$  at different energies  $W_\mu$  from zero upwards. Our view is that there are three types of cone in operation, each one of which in the absence of the others would give a curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  similar in shape to curve 2 of fig. 36. In the top right-hand quadrant of fig. 44 the three curves  $R$ ,  $G$  and  $B$  represent the variation of  $\log_{10} U_\lambda$  with  $\log_{10} W_\mu$  for the red, green and blue cones respectively, acting alone. The position of each curve with respect to the axes of  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  is determined by the wave-lengths  $\lambda$  and  $\mu$ . When  $W_\mu$  becomes very small and  $\log_{10} W_\mu$  tends to minus infinity, each of the three curves flattens out and  $\log_{10} U_\lambda$  becomes constant. This constant value corresponds to the absolute threshold of the particular type of cone and depends only on the wave-length of the test stimulus. We have defined  $b_\lambda$  to be the reciprocal of the absolute threshold of the blue cones for a test stimulus of wave-length  $\lambda$ , so that curve  $B$  must flatten out to the value  $\log_{10}(1/b_\lambda)$  or  $\{-\log_{10} b_\lambda\}$ . Similarly, curves  $R$  and  $G$  must flatten out to  $\{-\log_{10} r_\lambda\}$  and  $\{-\log_{10} g_\lambda\}$  respectively. In the top left-hand quadrant of fig. 44,  $\{-\log_{10} b_\lambda\}$ ,  $\{-\log_{10} g_\lambda\}$  and  $\{-\log_{10}(r_\lambda)\}$  are plotted against  $\lambda$ , the vertical scale being precisely the same as the vertical scale of  $\log_{10} U_\lambda$  on the other side of the diagram. The three curves are simply curves 1, 3 and 4 of fig. 40, turned upside down. Thus if we take a particular wave-length of the test stimulus, say  $\lambda = 470 \text{ m}\mu$ , then the vertical line through 470 on the axis of  $\lambda$  will intersect the curve of  $\{-\log_{10} g_\lambda\}$  at the value to which curve  $G$  must flatten out when  $\log_{10} W_\mu$  tends to minus infinity. The values at which curves  $R$  and  $B$  flatten out are similarly determined.

It remains to fix the position of curves  $R$ ,  $G$  and  $B$  with respect to the axis of  $\log_{10} W_\mu$ . It will be recalled that  $R_\mu$ , was defined as the reciprocal of the energy of the  $10^\circ$  patch necessary to raise the l.b.i. of the red cones to ten times their absolute threshold. That is to say, to cause such an increase the  $10^\circ$  patch must have energy  $W_\mu$  such that

$$\log_{10} W_\mu = \log_{10}(1/R_\mu) = \{-\log_{10} R_\mu\}.$$

In the lower right-hand quadrant of fig. 44  $\{-\log_{10} R_\mu\}$  is plotted against  $\mu$ , the horizontal scale being precisely the same as the horizontal scale of  $\log_{10} W_\mu$  at the top of the figure. Thus if we take a particular wave-length of the  $10^\circ$  patch, say  $\mu = 620 \text{ m}\mu$ , the horizontal line through 620 on the axis of  $\mu$  must intersect the curve of  $\{-\log_{10} R_\mu\}$  at a point  $P$  whose coordinate on the scale of  $\log_{10} W_\mu$  will be the value of  $\log_{10} W_\mu$  necessary to raise the l.b.i. of the red cones to ten times their absolute threshold. The point on the curve  $R$  at which the l.b.i. is ten times the absolute threshold is marked

with a dot  $C$  and the position of curve  $R$  is fixed by bringing this dot to lie on the vertical line through  $P$ .

A similar procedure fixes the position of curves  $G$  and  $B$  except that for  $B$  if  $\mu$  exceeds  $570 \text{ m}\mu$  the curve must be modified to the form of curve 3 of fig. 36 after its position has been determined by the above method. This modification of curve  $B$  is necessary in the example illustrated in fig. 44.

It is clear that with the aid of the diagram of fig. 44 the three curves  $R$ ,  $G$  and  $B$  can be drawn in for any wave-lengths of test stimulus and central field.

It has been tacitly assumed that both test stimulus and the conditioning stimulation enter at  $d = d_m$ . For other points of entry of the test stimulus the curves  $R$ ,  $G$  and  $B$  must be shifted upwards parallel to the axis of  $\log_{10} U_\lambda$  by amounts  $p_{\lambda b}(d - d_m)^2$ ,  $p_{\lambda g}(d - d_m)^2$  and  $p_{\lambda r}(d - d_m)^2$  respectively. For other points of entry of the central field the component curves must be shifted to the right parallel to the axis of  $\log_{10} W_\mu$  by amounts  $P_{\mu b}(d' - d_m)^2$ ,  $P_{\mu g}(d' - d_m)^2$  and  $P_{\mu r}(d' - d_m)^2$ .

The derivation of the resultant curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  for foveal vision now depends on a method for determining  $\log_{10} U_\lambda$  at each value of  $\log_{10} W_\mu$  from the values of  $\log_{10}(U_\lambda)_b$ ,  $\log_{10}(U_\lambda)_g$  and  $\log_{10}(U_\lambda)_r$  given by curves  $B$ ,  $G$  and  $R$ . We have been able to proceed without defining precisely how this is to be done by assuming that  $\log_{10} U_\lambda$  will not exceed the smallest of  $\log_{10}(U_\lambda)_b$ ,  $\log_{10}(U_\lambda)_g$  and  $\log_{10}(U_\lambda)_r$  and will be actually equal to it except when the smallest and the next smallest have about the same value. Further work on this point is necessary.

For parafoveal vision it has been shown that the rods give rise to a curve of  $\log_{10} U_\lambda$  against  $\log_{10} W_\mu$  which has the shape of curve 1 of fig. 36 and whose position with respect to the axes is fixed, in any given case, by the values of  $\log_{10} s_\lambda$  and  $\log_{10} S_\mu$ . The relation between  $\log_{10} U_\lambda$  and  $\log_{10} W_\mu$  given by the parafoveal cones cannot be represented in this simple way. By assuming that the parafoveal cones, like the foveal cones, consist of three species, each with its own spectral sensitivities to test stimulus and conditioning stimulation, we can explain the parafoveal measurements. If the parafoveal and foveal cones had identical properties it would only be necessary to add the rod curve as a fourth component curve in fig. 44 and determine the resultant of the four component curves so obtained. When this is done the resultant curves for different combinations  $\lambda$  and  $\mu$  reproduce the main features of the experimental curves for parafoveal vision, and show anomalies of the kind noted at the end of § 7. However, the agreement in the cone regions of the curves is not quantitative, and we must conclude that there are significant differences in the spectral sensitivities

of the parafoveal and foveal cones of corresponding type. On the other hand, the shape of the individual cone component curves is probably the same for both parafovea and fovea. Thus, a tracing of curve 2 of fig. 30 can be fitted over the cone sections of curves I and II in fig. 19.

Evidence for the existence of three types of cone and a first crude determination of the unique set of spectral sensitivity curves for these types have been obtained in this investigation by a method completely independent of colour-matching measurements. The precise connexion between the three mechanisms demanded by colour-matching experiments and the three types of cone assumed here must be close but it remains to be elucidated.

The writer has much pleasure in acknowledging the assistance rendered by Mr Gordon-Smith and Mr Dew in carrying out the measurements described. His thanks are also due to Professor Hartridge for helpful criticism of the paper.

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#### SUMMARY

A light ray terminating on a given point of the retina has different visual effects depending on its direction of incidence on the retina. The direction of incidence can be varied by sending the ray into the eye through different points of the pupil opening. The primary object of this work was to determine the effect of direction of incidence on threshold sensitivity but the results obtained cover the wider problem of the dependence of threshold sensitivity on the condition of stimulation of the retina. The test stimulus was a patch of light of diameter  $1^\circ$  and of wave-length  $\lambda$ , exposed for a fraction of a second every few seconds.  $U_\lambda$ , the smallest perceptible intensity of the test stimulus, was measured in energy units, the sensitivity then being defined as  $1/U_\lambda$ . With parafoveal vision of the test stimulus by the dark-adapted eye it was found that direction of incidence had little effect on sensitivity when  $\lambda$  was less than  $580\text{ m}\mu$  but a pronounced effect for longer wave-lengths. The dark-adapted fovea gave a pronounced directional effect at all wave-lengths.

The condition of the retina was modified by making the subject view a patch of light of diameter  $10^\circ$  (wave-length  $\mu$ , and intensity  $W_\mu$ ) at whose centre the test stimulus was applied as an *additional* stimulus. For parafoveal vision, the directional effect showed a marked increase in passing

from low to high intensities, and the curve connecting the threshold value  $U_\lambda$  and the intensity of the conditioning field  $W_\mu$  showed a corresponding change of law. These two connected effects are attributed to a change from rod vision at low intensities to cone vision at high intensities, the rods and cones being assumed to have different directional properties. For foveal vision, which involves the cones only, somewhat similar effects were observed and are explained by assuming three types of cones for which the relative spectral sensitivities are roughly determined. Normally the threshold value increases proportionally with the intensity of the conditioning field at high intensities (Weber's Law) but a striking deviation from this rule was observed for a blue test stimulus on a red conditioning field. Increase in the intensity of the conditioning field beyond a certain value produced no corresponding increase in the threshold value. This result is ascribed to a special property of the "blue" cones. In the present investigation the hypothesis of three types of cone whose properties have been approximately determined has been developed from measurements which are completely independent of colour-matching data.

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# A reinvestigation of turacin, the copper porphyrin pigment of certain birds belonging to the Musophagidae

BY CLAUDE RIMINGTON

*From the National Institute for Medical Research*

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## INTRODUCTORY

The first careful examination of the red pigment occurring in the wing feathers of certain turacos, African birds belonging to the family Musophagidae, N. O. Scansores, was made by Church in 1869, and he it was who proposed the name "Turacin" for this pigment. His observations were extended in 1892, and since then the subject has received study at the hands of Laidlaw (1904) and Fischer and his associates. Fischer and Hilger (1924) reported that turacin was identical with the copper complex of uroporphyrin I.

The system of nomenclature developed by Fischer is based upon differences in regard to the positions of the substituent groups upon the pyrrole nuclei comprising the porphine ring system. In the case of the actioporphyrins, there are present four methyl and four ethyl groups which may be arranged in such a manner that four possible isomeric actioporphyrins result. Fischer has named these actioporphyrins I, II, III and IV, and each may be regarded as the parent substance of a potential series of porphyrin pigments. Replacement of the four ethyl groups by propionic acid residues gives rise to the coproporphyrins which are thus tetracarboxylic acids. When, in addition, four acetic acid residues take the place of the methyl groups, the uroporphyrins result, possessing eight carboxyl groups in the molecule. These relationships are illustrated by the formulae for coproporphyrins I and III, and for uroporphyrin I, reproduced in the accompanying figures (see figs. 1-3).

So far, only porphyrins belonging to the isomeric series I or III have been found in nature. For a more detailed discussion of the occurrence in nature



of porphyrins of the two series, the publications of Fischer and his co-workers or the paper by Rimington (1936) may be consulted. Haemoglobin, the bile pigments, and the prosthetic groups of catalase and cytochrome C, are all derivable from series III, and these isomers have come to be regarded as "physiological". On the other hand, derivatives of series I,

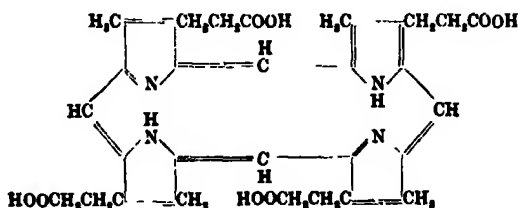


FIG. 1. Coproporphyrin I. Ester m.p. 251° C.

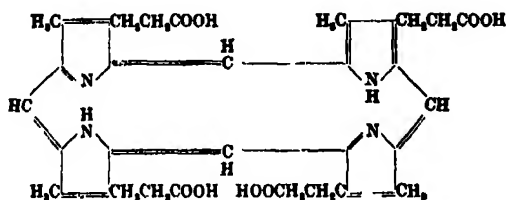


FIG. 2. Coproporphyrin III. Ester m.p. 145° and 172° C.

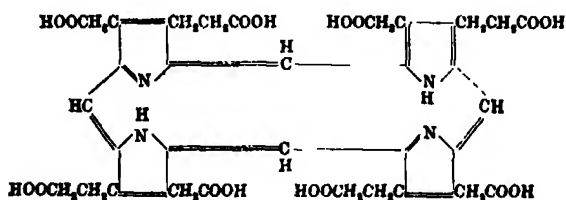


FIG. 3. Uroporphyrin I—probable structure.

such as coproporphyrin I and uroporphyrin I, which are excreted in relatively large quantities in congenital porphyria, have, by contrast, been regarded as "pathological" or "abnormal" products. The rigidity of this distinction has been weakened by recent developments, and it has now become clearly untenable. Rather must it be recognized that in many instances the body elaborates both isomeric types, and pathological conditions are characterized chemically by an alteration in the ratio of I to III series isomers, as well as by enhanced total excretion of porphyrin.

Thus, it was shown by Rimington (1936, 1937), investigating bovine congenital porphyria, and by Fischer and Hoffmann (1937) in the case

of material from the human congenital porphyrinuric "Petty", that the uroporphyrin occurring in bones and urine in this anomaly is not pure uroporphyrin I, as previously supposed, but a mixture of uroporphyrin I and uroporphyrin III, in which the I series isomer predominates. Similarly, in congenital porphyrinuric bovines, the urinary coproporphyrin was shown to consist of a mixture of the I and III series pigments (Rimington and Roets 1937).

The ratio of the two isomers, one to the other, has been determined for a number of affected bovines (unpublished data of Rimington and Roets), and, although this is not constant from animal to animal, it is clear that the proportion of coproporphyrin III in the total mixed excretion is by no means negligible (e.g. 3.7 % in one case).

A coproporphyrin from normal human urine was first obtained in crystalline condition by Fink and Hoerbuerger (1934), who identified it as coproporphyrin I, but quite recently Grotepass (1938) has reinvestigated this question of the normal urinary pigment and found that, here again, coproporphyrin III accompanies coproporphyrin I, in this case in nearly equal quantity. So far, the principle of duality has not been extended to the haemoglobins, both normal haemoglobin and that of congenital porphyrinurics being regarded as a series III derivative. However, it may be recalled that Fischer and Kirmann (1929), when investigating this problem by means of conversion of the prosthetic group into the corresponding mesoporphyrin, and isolation and identification of the crystalline ester of the latter, experienced considerable difficulty in obtaining a mesoporphyrin ester with the right melting-point. Nearly all their preparations melted some 10° or so lower than does pure Meso IX ester, and the possibility would seem to be not excluded that further refinements of technique may yet show that two isomeric types of haemoglobin circulate in mammalian blood.

The chemical evidence adduced by Waldenström (1935, 1936), and by Mertens (1936), during the past few years, that in acute idiopathic porphyrinuria uroporphyrin III is excreted, in contrast to the (preponderating) uroporphyrin I of congenital porphyrinuria, led at once to a re-orientation of ideas concerning the porphyrinurias and made it necessary to regard acute idiopathic porphyrinuria as a disease with a pathogenesis fundamentally distinct from that of porphyrinuria of the congenital type. Quite recently, however, Turner (1938) has described a case of acute idiopathic porphyrinuria, the urine of which contained both uroporphyrin I and uroporphyrin III, whilst Dobriner (1936) found both coproporphyrins I and III in the stools of a case of chronic porphyria with no

history of poisoning by lead or sedative drugs. It is clear that the interpretation of the chemical findings is beset with difficulty; the more closely excretory pigments are studied in any condition, the more frequently does it appear that both isomeric types are present.

The work now reported was commenced in the summer of 1937, when what might be termed the "unitarian doctrine" of the chemistry of congenital porphyrinuric pigments was being disproved by Fischer in Europe and by Rimington and Roets in South Africa. A reinvestigation of turacin seemed desirable in order to ascertain whether or not, in this case also, such a naturally formed pigment would be found to be associated with small quantities of its structural isomer. Fischer and Hilger (1924) had described the isolation from turacin of uroporphyrin I with ester m.p. 295°, similar to that found by Fischer in "Petry" urine, but at that date the existence of porphyrin duality had not been clearly recognized, and uroporphyrin III had not then been described. It was thought possible, therefore, that small quantities of the more soluble III isomer might have been overlooked in working up the experimental material.

Before entering into a detailed description of the experiments, however, a short summary may be presented of our knowledge concerning this interesting pigment, particularly since over half a century has elapsed since Church's publications, which may still be read as classics.

#### PREVIOUS CHEMICAL INVESTIGATIONS UPON TURACIN

Church (1869), whose interest in the subject was stimulated by Tegetmeier, recalls that a Mr Ward of Wigmore Street had noticed the ease with which the red pigment of turaco feathers may be leached out when the coloured portion is placed in water. It was observed later that a little soap or alkali markedly facilitated solution. Dr B. Hinde, principal military medical officer at Bathurst, Gambia (1865), sent home to a friend in Ireland a pair of violet plantain-eaters (*Musophaga violacea*), but after a few days in captivity, the birds being "provided with ample space and all appliances for cleanliness", the crimson colour of the wing feathers had entirely disappeared, much to the disappointment of the new owner, who naturally thought Dr Hinde had been imposed upon. A third bird, so young as to be only partly fledged, was procured, however, and as soon as the adult plumage had developed, it was released in the enclosure with identical result.

Church in his first paper (1869) records the detection of turacin in twelve different species of the Musophagidae and its isolation from four by

means of solution from the defatted feathers in dilute alkali, precipitation with acid, etc. The absorption spectrum of the pigment and its properties are recorded, including elementary analyses and the detection of copper as an integral constituent of the pigment molecule. The resemblance shown by turacin to haematin was perceived by Church, but he refrained from regarding it as a blood pigment containing copper instead of iron. The close relationship existing between the two was demonstrated experimentally by Laidlaw in 1904.

In his second publication of 1892 Church records instances of turacos in captivity having moulted and renewed their crimson plumage, without having had access to any source of copper other than that provided by their vegetable diet consisting chiefly of bananas. "Decided traces" of copper were detected in the ash left by bananas when incinerated. He recalls the discovery by Fredericq (1878) of a second copper-containing pigment in nature, the now familiar haemocyanin, and mentions that Lupton (1873) found copper in the ash of green feathers from an Australian love-bird, *Melopsittacus undulatus*.

The number of turacos containing turacin was at this time advanced by Church to eighteen,\* and extensive notes were given concerning the geographical distribution of the birds. The pigment itself was subjected to a careful spectroscopic examination (with the aid of MacMunn), and the figures given for the positions of the absorption bands are in exceedingly good agreement with modern determinations. Moreover, the partial removal of copper by concentrated sulphuric acid, to give a four-banded haematoporphyrin-like spectrum, was observed, and the name turacoporphyrin proposed for the product. Finally, many new analytical data for turacin were collected, the figures arrived at being not far from those now known to be demanded by its composition as a copper uroporphyrin complex.

It was this similarity with the composition of uroporphyrin which induced Fischer and Hilger (1923) to attempt the isolation of the pure ester, and to compare it with that derived from "Petry" urine. Removal of copper from the complex was effected by heating in a mixture of acetic and hydrobromic acids, but the ester of the free porphyrin failed to crystallize. In a later paper Fischer and Hilger (1924) record an alternative method which they eventually found to be successful; copper is removed by shaking the pigment in alkaline solution with sodium amalgam. It would appear

\* Based upon the classification in the *Brit. Mus. Cat. Birds*, 19, 1891. The family has since undergone extensive revision (see Sclater's "Systema Avium Æthiopicarum", 1924).

from a careful reading of this paper that considerable difficulties were still encountered, but a crystalline uroporphyrin ester was finally obtained having m.p.  $295^{\circ}$  C. and giving no depression with uro-ester from "Pettry" urine. The yield of turacin is given as between 0.1 and 0.15 g. from the feathers of a single bird, but the yield of crystalline uroporphyrin ester obtained is not recorded.

#### METHOD OF PRESENT INVESTIGATION

As final confirmation of the identity of any uroporphyrin, it is very desirable that the pigment should be transformed by decarboxylation into the corresponding coproporphyrin, since all four coproporphyrins and their esters have been synthesized, and their melting-points are known. A convenient means of bringing about this transformation has been discovered by Fischer and Zerweck (1924), the uroporphyrin ester being suspended in 1 % hydrochloric acid and heated under pressure for 3 hr. at  $180-190^{\circ}$  C.

The present investigation was commenced with about a dozen pigmented wing feathers from a living *Turacus corythaix*.<sup>\*</sup> The coloured portions were defatted by extraction with boiling ether and were then worked up according to the technique of Fischer and Hilger (1924), employing sodium amalgam for the removal of the copper. The final uroporphyrin methyl ester was dissolved in hot chloroform and boiling methyl alcohol added. The ester which separated, crystallized with difficulty, and the mother liquors were intensely coloured. As it seemed possible that some chloroform-soluble impurity was still present in the preparation, the entire pigment was saponified by alkali and, after several precipitations at the isoelectric point, the free uroporphyrin was again esterified and the ester crystallized as before. Once again the material crystallized with difficulty in the form of aggregations of extremely small needles suspended in an intensely coloured mother liquor. This behaviour is reminiscent of uroporphyrin III ester rather than of uroporphyrin I, which crystallizes from chloroform and methyl alcohol leaving a practically colourless mother liquor. Moreover, the melting-point of the ester derived from turacin was  $250^{\circ}$  and could not be raised substantially above this figure. The methyl ester of uroporphyrin I has m.p.  $302^{\circ}$ , that of uroporphyrin III m.p.  $255-7^{\circ}$ , when crystallized from methyl alcohol.

In order to obtain further results for comparison, another turaco was obtained from Dr Thomas of Onderstepoort Veterinary Laboratory. This

<sup>\*</sup> I am indebted to Dr Bigalke, Director of the Pretoria Municipal Zoological Gardens, South Africa, for this gift.

was a specimen shot in the Northern Transvaal for the South African Zoological Survey, and was afterwards identified for me by the British Museum (Natural History) as *Gallirex porphyreolophus*. A preparation of turacin from the feathers and of the derived uroporphyrin methyl ester led to a similar result, namely, a uroporphyrin ester melting at about 250° and having properties characteristic of the III series isomer. Since a quantitative separation of the coproporphyrin isomers and their individual identification is much more easily accomplished than that of the uroporphyrins, a larger quantity of turacin was prepared, derived from the feathers of these two birds (*Turacus corythaix* and *Gallirex porphyreolophus*), the corresponding uroporphyrin ester obtained, and this material subjected to decarboxylation forthwith. As the technique with subsequent specimens has differed in no material way, the experiment will be described in detail.

The coloured portions of the feathers were cut up and extracted in a Soxhlet apparatus with ether, then dried and weighed, and the pigment dissolved by soaking in several changes of 1 % ammonium hydroxide. The combined extracts, which exhibited the characteristic absorption bands of turacin at 562.6, 525.8  $m\mu$ , were filtered, and the pigment precipitated by addition of acetic or hydrochloric acid. The precipitated turacin was centrifuged off, washed with water, and the precipitation process repeated several times before finally drying *in vacuo*. Copper was removed by dissolving in dilute potassium hydroxide (20 c.c.) and adding 6.25 g. of 4 % sodium amalgam, in small portions at a time, and shaking vigorously after each addition. After some hours the colourless solution was filtered through a no. 50 Whatman paper, and the pigment reoxidized by bubbling a stream of air through the solution for 14 hr. Addition of acetic acid to pH 3.5-4.0 now precipitated the free uroporphyrin, which, after washing with water containing a little acetic acid, and reprecipitation, was dried and esterified by solution in methyl alcohol saturated with hydrochloric acid gas. After standing overnight the solution was diluted with methyl alcohol, filtered and the ester transferred to chloroform after copious dilution with water. The chloroform layer, washed with dilute sodium carbonate solution and then water, was filtered and evaporated to dryness in a tared basin, the weight of crude uroporphyrin ester being thus obtained.

The entire yield of uroporphyrin ester was now transferred to a hard glass combustion tube by means of chloroform, the solvent evaporated off, and about 30 c.c. of 1 % hydrochloric acid added. The tube was sealed and heated in a Carius furnace for 3 hr. at 196°. The resulting red, acid solution was filtered from some dark amorphous material (soluble in alkali to yield

a greenish brown solution without characteristic absorption bands), and shaken once with ether to remove a small quantity of brownish material. Fresh ether was then added, a little glacial acetic acid and sufficient saturated potassium acetate to make the solution neutral to congo red. Upon shaking, the coproporphyrin passed into the ether phase, which was separated, washed free from acetic acid, and evaporated to dryness. The porphyrin residue was esterified with methyl alcoholic hydrochloric acid as described for uroporphyrin, and the chloroform solution of the ester evaporated in a weighed dish. From 47.7 mg. of crude uroporphyrin ester there were obtained 23.6 mg. of crude coproporphyrin ester.

In order to effect the separation of coproporphyrin I and III, advantage was taken of the fact that the ester of coproporphyrin III is appreciably soluble in cold ether (more so in cold methyl alcohol), whilst that of coproporphyrin I is insoluble. Several workers in this field have employed methyl alcohol to separate the two isomers but in this laboratory cold, dry ether is preferred. The coproporphyrin III ester dissolves more slowly, but the pigment may be recovered directly from the solvent by concentration until crystallization commences, or to dryness, followed by chloroform-ether crystallization. A brownish pigment—presumably an oxidation product of coproporphyrin ester—and having an intense absorption band in the region of  $645\text{ m}\mu$ , is often present in small quantity at the end of a series of operations, and may seriously interfere with crystallization. It possesses the characteristics, however, of being very readily soluble in ether and also of possessing a high acid number (over 10), so that when operating in the manner outlined below it is easily removed. During crystallization from dry ether it is left almost entirely in the mother liquors. The crude coproporphyrin ester was washed with petroleum ether and then stirred with successive quantities of pure anhydrous ether (distilled over sodium). The first washings were deeply pigmented, but when solution became slower, the operation was stopped and the sparingly soluble residue dissolved in a little warm chloroform to which dry ether was added (fraction 5). No precipitate formed at room temperature, but in the ice chest a deposit of straight, prismatic needles, usually in clusters, came down, leaving a deeply coloured mother liquor. The crystals were centrifuged down, washed with a little anhydrous ether, which became appreciably coloured (fraction 4), and then dried. Yield 14 mg. The material proved to be pure coproporphyrin III methyl ester, m.p.  $153\text{--}153.5^\circ$ , remelt after cooling  $173\text{--}6^\circ$  (see fig. 4). All melting points were observed upon the electrically heated micro-melting point apparatus of Kofler, as supplied by A. Schenach, Innsbrück. A specimen of synthetic

coproporphyrin III tetramethyl ester from Professor H. Fischer softened at  $143^{\circ}$  and melted at  $150-3^{\circ}$ . A mixture with the ester isolated had m.p.  $150-1^{\circ}$ , remelt  $170-3^{\circ}$ . No coproporphyrin I ester could be detected in this material. The ether washing of these crystals (fraction 4) was shaken with a little 5 % hydrochloric acid which removed the ester, leaving in the ether a trace of the brown pigment with band at  $645\text{ m}\mu$ . By addition of potassium acetate, the material was now driven back from the acid to fresh ether, and this solution, after thorough washing, dehydrated with a pinch

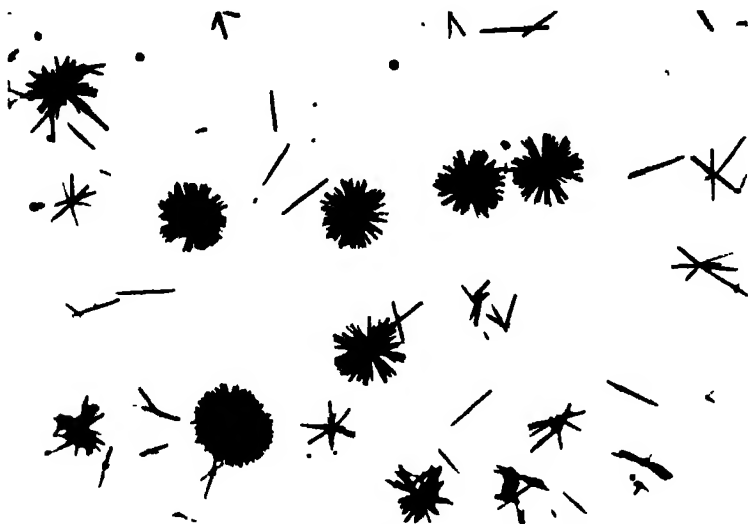


FIG. 4. Coproporphyrin III tetramethyl ester, m.p.  $153-153.5^{\circ}\text{C}$ , remelt  $173-6^{\circ}\text{C}$ , by decarboxylation of the turacin obtained from *Turucus corythaix* and *Gallirex porphyreolophus* (mixed sample).  $\times 120$ .

of anhydrous sodium carbonate, filtered and allowed to evaporate. The residue consisted of beautifully formed crystals of coproporphyrin III ester, m.p.  $150^{\circ}$ , remelt  $174-6^{\circ}$ .

The deeply coloured mother liquor of the main crystallization (fraction 5) was added to the ether washings of the crude esters and purified by transference to hydrochloric acid, firstly 0.5 %, which removed a trace, followed by 5 % acid and recovery as outlined above. Fractions 1 and 2 were obtained having m.p.  $147-9^{\circ}$ , remelt  $173-5^{\circ}$ , and  $149-51^{\circ}$ , remelt  $174-6^{\circ}$  respectively.

Since all these smaller fractions consisted of coproporphyrin III ester, they were combined and recrystallized, yielding 4 mg. with m.p.  $146-8^{\circ}$ , remelt  $172.4^{\circ}$ , thus bringing the total yield of pure crystalline material to



18 mg. or 76.3 % of the crude coproporphyrin ester obtained from the decarboxylation. The spectrum in ether measured with a simple direct-vision spectroscopie was 625, 588-570, 542-530, 512-485  $m\mu$ . Microanalysis confirmed the identity with coproporphyrin III methyl ester. Since such small amounts of material could be spared, only single determinations were possible. It will be recalled that a tendency to incomplete combustion is apt to afford somewhat low carbon figures for porphyrin materials.

## Microanalysis

3.870 mg. subs. gave 9.395 mg.  $\text{CO}_2$  and 2.27 mg.  $\text{H}_2\text{O}$   
 2.955 mg. " " 3.930 mg. AgI  
 2.932 mg. " " 0.213 c.c. of  $\text{N}_2$  at 23° C and 754 mm.

	C	H	N	$\text{CH}_3\text{O}$
Found	66.21	6.56	8.3	17.57
$\text{C}_{40}\text{H}_{46}\text{O}_8\text{N}_4$ (710.41) requires	67.56	6.53	7.9	17.47

These results were entirely unexpected, as it has been customary to regard turacin as a pigment of the series I type. At the most it was hoped that traces of coproporphyrin III might be found, together with a larger quantity of coproporphyrin I. In order to be certain that no unforeseen complication had arisen during the operations of decarboxylation, etc., a specimen of a known uroporphyrin I ester, m.p. 293°, from bovine congenital porphyrinuric bone was decarboxylated and worked up in the same way. The coproporphyrin ester obtained crystallized at once from hot chloroform-methyl alcohol mixture in the typical long curved needles of coproporphyrin I ester and had m.p. 251-2°.

## RESULTS OBTAINED WITH DIFFERENT SPECIES

It was next considered possible that different isomeric turacins might be present in different turacos, and a careful search was therefore made to find which species Fischer and Hilger used in their original investigations. Although there are frequent references in their two papers (1923, 1924) to "Turacos" and "Helmvögel" (different individuals or species?), I have only been able to find one instance in which a systematic name is quoted - - on p. 64 of the 1924 article it is stated that a turacin derived from *Turacus zenkeri* was used to prepare the crystalline uroporphyrin ester which had m.p. 293°. *T. zenkeri* is now regarded as a race of *T. persa*.

It seemed that an investigation was desirable of as many of the turacin-bearing species as possible, since these birds have been repeatedly instanced as producing a "pathological" pigment—in fact as suffering from a physiological porphyria of the type I or congenital type.

Through the courtesy of the Trustees of the British Museum (Natural History) I was supplied with one specimen each of eleven of the seventeen species of these very rare birds known to contain a red pigment. The members not examined include *Musophaga violacea* (West Africa), *Proturacus bannermanni* (Cameroons), *Turacus schuetti schuetti* (Angola and Belgian Congo), *T. fischeri* (coast of East Africa), *T. erythrolophus* (Angola), and *T. reichenowi* (Portuguese East Africa). I have, however, been able to examine one species at least of each of the above genera, except *Proturacus*. The genera *Corythaecola*, *Crinifer*, *Corythaixoides* and *Gymnoschizorhis* of the Musophagidae are reported not to contain the pigment (Selater 1924).

Since only limited quantities of material were available, great care had to be exercised in order to obtain sufficient pure pigment for the final characterization; a micro-technique of manipulation was adopted throughout. The method of preparation and working up of the pigment to the final coproporphyrin ester differed in no essential from that already described. The results obtained are presented in the following table (see Table I).

It is clear from the above records that, from each bird investigated, a pigment corresponding to coproporphyrin III methyl ester was eventually obtained, and in no case could the presence be detected of the ester of coproporphyrin I. Only in a few instances was any material difficultly soluble in ether encountered in the crude coproporphyrin ester mixture, and, when attempts were made to crystallize these traces from chloroform-methyl alcohol, the resulting material proved to be either coproporphyrin III ester or an amorphous reddish brown substance, resembling in its properties the ester of uroporphyrin III (cf. note upon *Turacus persa zenkeri*).

I am unable to explain the divergence of my results from those of Fischer and Hilger, but the following observations may not be out of place.

According to their first paper on turacin, Fischer and Hilger (1923) were unable to crystallize the ester of the uroporphyrin obtained by removal of copper from the feather pigment, although it was precipitated from hot chloroform-methyl alcohol solutions in a form resembling that of uroporphyrin ester, and exhibited the correct spectrum when in chloroform solution. Attempts to prepare the copper complex of the ester in crystalline form, by methylation of turacin, were likewise fraught with difficulty, and successful in only one instance. The product, however, was accompanied by tarry material which precluded a melting-point determination. In considering these results, Fischer and Hilger entertained the possibility that a mixture of isomers might be present, but inclined rather to the view that failure to obtain crystalline products was due to the poor condition of the starting material.

TABLE I

Name	Wt. pigmented feathers used g.	Wt. uroporphyrin ester mg.	Wt. total coproporphyrin ester mg.	Melting-point of recryst. coproporphyrin ester
<i>Gallirex porphyreolophus</i>	0.37	28.8 (m.p. 250-4°)	8.6	148°, remelt 175-6° (wt. 4.8 mg.)
<i>Musophaga rossae</i>	0.63	52.4 (m.p. 205-15°)	8.6	147-8°, remelt 170-1°
			Additional traco	145°, remelt 172-5°
<i>Ruwenzorornis johnstoni johnstoni</i>	0.42	25.1 (m.p. 215-25°)	12.0	144-6°, remelt 172°
<i>Turacus schalowi marungensis</i>	0.52	30.0	27.8*	143°, remelt 162°
<i>Turacus corythaix</i>	0.33	20.0 (m.p. 250-4°)	7.4	149°, remelt 175° (wt. 7.2 mg.)
<i>Turacus persa zenkeri</i>	0.65	38	17.0*	147-8° (3.6 mg.), 172° (additional 1.2 mg.)
<i>Turacus macro-rhynchus verreauxi</i>	0.60	35.4 (m.p. 210°)	6.0	172-4°, after recryst. 149°, remelt 175-6°
<i>Turacus leucotis leucotis</i>	0.67	29.4	19.0*	163-5°, remelt 172-3° (wt. 4.8 mg.)
<i>Turacus leucolophus</i>	0.51	26.7	10.1*	139-40°, remelt 167-9° (wt. 2.5 mg.)
<i>Turacus livingstoni livingstoni</i>	0.50	25.5	10.2	145°, remelt 168°, recryst. 172-3°
<i>Turacus hartlaubi</i>	0.47	26.8	12.2	164-7°, remelt 170-3°
Residual mother liquors combined			1.5	148°, remelt 174-5°

\* Owing to incomplete decarboxylation, some unchanged uroporphyrin ester, insoluble in ether, remained in this fraction. It was removed by saponifying the esters and extracting the coproporphyrin with ether in the usual way. The coproporphyrin was then re-esterified and treated with anhydrous ether for the separation of any coproporphyrin I ester that might be present.

In their later communication (1924), attempts are again recorded to prepare the methyl ester of turacin by the use of potassium methylate and potassium hydroxide. Only once were crystals obtained and these were too small to permit of exact identification. More success followed the preparation of the free uroporphyrin and its methyl ester. Copper was removed from 0.1 g. of turacin (derived from *Turacus zenkeri*) by means of sodium amalgam, and the resulting uroporphyrin esterified by means of alcoholic hydrochloric acid. The product was crystallized from chloroform-methyl alcohol and washed with methyl alcohol, after which it melted at 205°. Unfortunately no yield is recorded. It seems to me possible, in seeking an explanation of the divergence between the present results and those of Fischer

and Hilger, that these workers may have had a mixture of the two isomeric uroporphyrin esters but, by crystallization from the solvents mentioned and repeated washing with methyl alcohol, have eliminated the relatively much more soluble uroporphyrin III methyl ester. This isomer can be extracted gradually from a mixture by stirring with methyl alcohol, although the process is very tedious. It is conceivable that, in these circumstances, a small quantity of relatively pure uroporphyrin I ester might have been obtained.

In my own experiments I was obliged to work with very small quantities of material, but, as has been pointed out earlier, I deliberately refrained from any attempt to recrystallize the uroporphyrin ester until pure. Care was taken rather to include every trace of the crude uroporphyrin in the decarboxylation mixture, and an attempt to separate series I from series III isomers was made only when the entire pigment had been transformed into the corresponding coproporphyrin ester. The loss during decarboxylation is considerable and the yield of coproporphyrin ester small, as found by Fischer and other workers, but uroporphyrins I and III react similarly in this respect. Not the slightest difficulty was encountered in obtaining a well-crystallized coproporphyrin I ester of m.p.  $251-2^{\circ}$  from an authentic specimen of uroporphyrin I ester (m.p.  $293^{\circ}$ ).

The purity of the final coproporphyrin ester III obtained in each case can be judged from its melting-point. Fischer and Hierneis (1931) have shown, in a thorough investigation of the behaviour of the ester of coproporphyrin III, that this substance may crystallize in either the low ( $145^{\circ}$ ) or high ( $172^{\circ}$ ) melting-point form, or the preparation may show a melting-point intermediate between these figures. On heating above the first melting-point, cooling and then again recording the melt, the fusion point is now found to be raised to the higher figure, provided the specimen is pure. When, however, coproporphyrin I ester was added to the III isomer, the melting-point could not be raised above  $135^{\circ}$  by this process of fusion and cooling. Referring to the figures recorded in the present communication, it will be seen that in the majority of instances, the remelting-point of the esters was  $170^{\circ}$  or over; only in a few instances was I unable to secure a final melt better than  $165^{\circ}$  (e.g. *T. leucolophus*, *T. schalowi*). However, a rise from the first fusion point was invariably recorded. It is not impossible that these preparations contained very small quantities of the I isomer, but such was not to be detected or eliminated by fractional crystallization, and I have no alternative but to conclude that the turacins which I prepared and examined were, substantially, series III pigments of a configuration similar to that of haemoglobin.

I am greatly indebted to those who made this investigation possible by kindly supplying me with valuable material, namely, Dr Bigalke, Director of the Pretoria Municipal Zoological Gardens, for a gift of feathers from *Turacus corythaix*; Dr Thomas of Onderstepoort Veterinary Laboratory and the South African Zoological Survey for a specimen of *Gallirex porphyreolophus*; and the Trustees of the British Museum, for very kindly putting at my disposal one specimen of each of the remaining species examined. I also wish to thank Mr J. Macdonald of the ornithological section, British Museum (Natural History), for his kindness in identifying the specimen of *Gallirex porphyreolophus* and for allowing me to examine the collection at the Museum.

My thanks are also due to my technical assistant, Mr A. W. Hemmings, for his careful and skilful aid in the preparation of the materials and the conduct of the chemical operations involved.

#### SUMMARY

Whilst investigating turacin, prepared from the wing feathers of *Turacus corythaix*, for possible traces of the III series isomer, it was found that the coproporphyrin obtained by decarboxylation of the uroporphyrin ester appeared to consist almost entirely of coproporphyrin III. A thorough examination has therefore been made of the pigment derived from eleven different species of the Musophagidae, which included at least one representative of every turacin-bearing genus, except *Proturacus*.

The turacin was extracted from the defatted feathers and converted into the corresponding uroporphyrin ester. No attempt was made to obtain a pure uroporphyrin ester at this stage. Since the isomeric coproporphyrins are more easily separated than are the uroporphyrins, the entire yield of crude uroporphyrin was decarboxylated in dilute acid under pressure and the resulting coproporphyrin fraction esterified. The coproporphyrin methyl ester so obtained was fractionated by a method which serves to separate the series I from the series III isomer. In every case crystalline coproporphyrin III methyl ester, with the correct double melting-point, was obtained, but in no instance was I successful in isolating or detecting the presence of any coproporphyrin I methyl ester. These turacins would appear, therefore, to be, substantially, derivatives belonging to series III, to which the normal porphyrin pigments of animals and plants predominantly belong.

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# The role of blue-green algae in nitrogen fixation in rice-fields

By P. K. DE

*Queen Mary College, University of London*

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The problem, with which this paper deals, arose out of the observation that in India rice can be grown on the same land for long periods without the addition of manure to the soil (Howard 1924, p. 114).

Three distinct periods are distinguishable in Indian rice fields, viz.

(1) Waterlogged period (from transplantation up to harvest time), in which there are a few inches to several feet of water above the soil and which is distinguished by the growth of abundant algae.

(2) The dry period, which follows in winter after harvest and during which conditions remain very suitable for microbiological activity.

(3) The desiccation period, commencing after winter when the soil temperature frequently exceeds 50° C.

The ability of rice to grow, year after year, in the absence of manure, might either be the result of the fixation of nitrogen in the soil, or the rice plants themselves might harbour a nitrogen-fixing symbiont after the manner of the Leguminosae. In the latter connexion it is noteworthy that Sen (1929) has demonstrated the occurrence of a nitrogen-fixing bacterium within the root of the rice plant, while Viswanath (1932) has obtained indications that rice plants possess the power of assimilating elementary nitrogen. In the waterlogged period conditions are unfavourable for certain bacteria like *Azotobacter* (De and Bose 1938) which are unlikely to be very active at this time.

The abundant growth of algae in the first period is beneficial in aerating the upper layers of the submerged soils (Harrison and Aiyer 1914). A number of experiments, carried out in India, to ascertain whether fixation of nitrogen takes place at these times suggested the possibility of the algae present being involved and has led to the present investigation. In the experiments referred to, 15 g. samples of soils from different parts of India were covered with distilled water. In certain of these cultures kept in the

light, particularly in those having a high  $pH$ , abundant algal growth consisting mainly of blue-green algae appeared, and determinations made after three months showed a considerable increase in total nitrogen varying from 4 to 35 % (De 1936). The absence of fixation in the cultures kept in the dark implies that bacteria cannot alone be involved, while the fact that considerable fixation took place only in those producing abundant growth of blue-green algae suggests that these algae may play an important role. This is also indicated by the fact that, when a little of the algal growth from a Faridpur soil culture was kept in the nitrogen-free medium described on p. 128 (medium II) for 2 months, the algae grew abundantly with fixation of nitrogen (2.1–3.6 mg./100 c.c. of medium).

The fixation of nitrogen by blue-green algae was first examined by Bouillhac (1896)\* in species of *Nostoc* with negative results. Heinzo (1906), on the other hand, claimed to have obtained evidence of nitrogen fixation in a culture of *Nostoc* contaminated with a *Streptothrix*, which by itself exhibited no nitrogen fixation (cf. the criticism of Bristol and Page 1923, p. 379). Molisch (1926), utilizing two strains of *Nostoc* isolated from a state of symbiosis, found that they grew well in a nitrogen-free solution, and from this observation concluded that they were able to fix nitrogen. The growth of the algae, as shown by dry-weight determinations, was better in the nitrogen-free solution than in the presence of nitrate. Drewes (1928) isolated *Anabaena variabilis*, *A. sp.* and *Nostoc punctiforme* in pure cultures, the purity of which was tested by inoculation into four different bacteriological media. The algae were grown in nitrogen-free solutions and were found to fix nitrogen. Allison and Morris (1932) obtained *Anabaena variabilis* free of bacteria by exposure to the light of a quartz mercury vapour lamp for periods varying from 1 to 20 min. at a distance of 20 cm. Purity was tested for by inoculation into four commonly used bacteriological media, as well as by direct microscopic examination. When grown in Ashby's medium containing no nitrogen or sugar, an average fixation of 5 mg. of nitrogen was obtained in 100 c.c. of the media during a period of 75 days. Aeration with air containing 1 %  $CO_2$  or the addition of sucrose to the medium stimulated nitrogen fixation. More recently Allison and Hoover (1935), using a species of *Nostoc* isolated from soil and purified by the same method, found it to possess the capacity of fixing as much as 10–20 mg. of nitrogen per 100 c.c. of medium in 50–80 days, the medium containing neither fixed nitrogen nor a source of energy. When supplied with a suitable source of energy such as glucose, the alga fixed nitrogen in the dark. Vouk and Wellisch (1931) claim, without giving details, to have

\* For the earlier literature see also Schramm (1914).



isolated three species of symbiotic blue-green algae, and from their ability to grow in nitrogen-free solutions they conclude that they are able to fix nitrogen. Copeland (1932), in a very brief paper, reports that he has obtained from nitrogen-deficient warm springs *Oscillatoria princeps*, *O. formosa*, *Spirulina labyrinthiformis* and *Phormidium laminosum*, which were able to fix nitrogen. Gerhard Winter (1935) isolated two species of *Nostoc* and found that they did not fix nitrogen when grown in mineral salt solution without carbohydrates. In the presence of glucose and fructose a measurable amount of nitrogen was fixed, and more in the dark than in the light.

With the exception of Copeland (1932), therefore, all investigators have observed fixation of nitrogen only by *Nostoc* and *Anabaena*. One misses in these researches, however, adequate evidence as to freedom from bacteria on the part of the cultures. Molisch (1926), Vouk and Wellisch (1931), and Copeland (1932) do not state how the purity of the cultures was tested. Drewes (1928) and Winter (1935) used several bacteriological solid media and bouillon, but the evidence is not conclusive, since on solid media, in the presence of algae, there may be no visible growth of bacteria although present, while in bouillon certain nitrogen-fixing bacteria may not develop. A similar criticism applies to the researches of Allison and co-workers (1932, 1935).

My work falls into two stages, viz. (i) isolation of certain species of blue-green algae in a pure condition, free from bacteria and other contaminating organisms; (ii) investigation of the capacity of the pure cultures to fix nitrogen. The first stage, which occupied nearly a year, proved exceptionally difficult, owing to the copious mucilage which is produced by the algae involved and which not only provides a natural medium for the growth of bacteria, but renders their removal by purely mechanical means quite impossible.

#### ISOLATION OF THE ALGAE

Of the diverse soils, which had shown considerable nitrogen fixation, that from Faridpur was selected for detailed study. Growth composed of spores of *Anabaena*, *Phormidium foveolarum* and a green unicellular form, which had arisen from it in India, was brought to this country in sealed tubes. From this growth placed in Pringsheim's solution (p. 124) unialgal cultures were prepared of a species of *Anabaena* and of the *Phormidium*. A fresh series of cultures was also made from dried soil brought from India, which when covered with distilled water provided after some weeks a thick growth, composed mainly of species of *Anabaena*. The development

of Chlorophyceae was markedly reduced by transference to a nitrogen-free medium (p. 128) in which good growth of blue-green algae took place within a month.

Before attempting to obtain unialgal cultures, the growth in different solutions, viz. those of Benecke, Geitler, Maertens, Brunnthaler and Pringsheim (cf. Kufferath 1930), was investigated. The solutions of Geitler, Maertens and Brunnthaler all proved unsatisfactory both as regards rapidity and amount of growth. In Pringsheim's solution the ultimate bulk of the growth was considerable, but it developed slowly. The initial growth was very rapid in Benecke's solution, but after a short time the cultures became almost colourless. This was found to be due to an increase in acidity of the medium (initial pH 6.8, final pH 5.2) resulting from a more rapid absorption of ammonium ions than nitrate ions. To neutralize the acidity the amount of phosphate in the medium was increased to 1 g./l. In this solution the growth looked healthier, but the algal cells were abnormally elongated, and when a little of the material was subcultured in the same medium (Benecke's solution) growth took place only very slowly. The filaments of *Anabaena* grown in Benecke's solution were almost devoid of heterocysts. The presence of ammonium nitrate in the Benecke's solution being thus clearly disadvantageous, the medium was modified for my purposes by substituting potassium nitrate for ammonium nitrate. In this modified solution growth was nearly as rapid as in the original Benecke, and the *Anabaena* filaments showed no abnormalities. With certain modifications, it was used in all the subsequent experiments. Its composition is as follows:  $\text{KNO}_3$  0.2 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g.;  $\text{K}_2\text{HPO}_4$  0.2 g.;  $\text{CaCl}_2$  0.1 g.;  $\text{FeCl}_3$  (1 %) 2 drops; water (pyrex distilled) 1000 c.c.

In some experiments sterilized soil extract\* was used as the base. Not only does such a medium afford rapid and abundant growth, but the algae remain healthy for a long period rendering frequent subculturings unnecessary. Soil extract was not employed, however, until the cultures had been obtained in a pure condition.

In obtaining unialgal cultures the following method was finally adopted: pieces of the material are streaked over the surface of several agar plates by means of a platinum loop, and these are then exposed to light until at least some show good growth (2-3 weeks). Under the microscope numerous filaments radiate from the points of inoculation, and single healthy ones

\* The soil extract used throughout this investigation was prepared by autoclaving for half an hour at 20 lb. pressure 1 kg. of soil with 1000 c.c. of tap water, with the addition of a little calcium carbonate. The extract was filtered, again autoclaved, and then allowed to stand for a few days. The clear supernatant liquid was carefully decanted off and preserved after sterilization in the autoclave.

can be selected and their positions marked with Indian ink. Portions of the agar including such marked areas are then cut out, again examined microscopically to make sure that only one filament is included and transferred to the liquid medium (p. 124). One or other plate always provided filaments suitable for isolation by this method, which enabled me to obtain five species of *Anabaena* and *Phormidium foveolarum* in unialgal culture.

The forms, for the determination of which I am indebted to Professor F. E. Fritsch, are *Anabaena gelatinosa* n.sp., *A. naviculoides* n.sp., *A. variabilis* Kütz. var. *ellipsozona* n.var., a form of *A. torulosa* (Carm.) Lagerh., *A. thermalis* Vouk var. *indica* n.var., and *Phormidium foveolarum* Gom.

In order to obtain bacteria-free cultures of these forms, diverse methods have been tried, using unialgal cultures of *Anabaena variabilis*. These included immersion in solutions of sea salt of different concentrations, exposure to drought, growth in a medium with different doses of thymol, treatment of the spores with a 0.2 % solution of mercuric chloride, exposure to ultra-violet light, and culture on silica gel plates. The last method, already used by Pringsheim (1913), was the only one that was successful; in the others either the bacteria were unaffected or the treatment proved lethal to the alga.

The silica gel was prepared by mixing equal volumes of hydrochloric acid (sp. gr. 1.1) and potassium silicate solution (sp. gr. 1.06); 40 c.c. portions of the mixture were placed in a number of 9 cm. Petri dishes. After 48 hr., when the gel had hardened, the plates were first washed in running tap water until free from acid and subsequently several times with boiled distilled water. Each plate was then impregnated with 4 c.c. of the following solution:  $\text{KNO}_3$  0.1 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g.;  $\text{K}_2\text{HPO}_4$  0.2 g.,  $\text{CaCl}_2$  0.1 g.;  $\text{FeCl}_3$  (1 %) 1 drop; water (pyrex distilled) 100 c.c.

Finally, the plates were exposed to a temperature of 60° C until the surface of the gel was fairly dry and then sterilized by heating in flowing steam for 15 min.

In the early experiments a rather heavy inoculum was used, but it soon became apparent that this did not lead to any marked reduction in bacterial numbers. The following method of inoculation was thereupon adopted. A little of the algal growth was vigorously shaken for half an hour with a little sterilized water in a test-tube closed with a rubber stopper. In this way the algal filaments were broken into fragments and the bacteria tended to be washed out of the mucilage. The number of fragments in several loopfuls of this suspension was counted under low power and the suspension was diluted with water so that a loopful contained

only 3-5 fragments. A loopful of the diluted suspension was transferred to the centre of a silica plate and spread over the surface by means of a bent glass rod. After this the plates were exposed to light in a greenhouse. After the appearance of colonies a little of each was transferred to one of the soil-extract-nitrate-cane-sugar-agar plates described below, and, if found contaminated, fresh subcultures on silica gel were made as above and the process repeated. When one or more colonies were obtained, which appeared to be pure when tested by the above method, they were transferred to a culture medium and allowed to grow for some time. They were finally tested for purity by the methods described below. In this way *Anabaena naviculoides* and *A. variabilis* were obtained in pure culture. The bacteria-free cultures of *A. gelatinosa* and *Phormidium foveolarum* were obtained by a slightly different method. These species readily form motile hormogonia which become distributed over the silica plate, so that isolated filaments or colonies are found on the plates far away from the point of inoculation. When such growths are transferred to the cane-sugar-nitrate plates, the alga grows at a much greater rate than the bacteria, so that some of the newly formed filaments outgrow the zone of bacteria. Further multiplication gives rise to a number of bacteria-free filaments.

It is necessary to apply a number of different tests to establish the purity of such algal cultures, since in the present investigation cultures apparently free from bacteria according to one test have shown evidence of contamination on being subjected to a different one.

The following four media (both solid and liquid) were used in testing the purity of the cultures: (1) nutrient agar; (2) egg-albumen-glucose-agar (Waksman 1922); (3) soil-extract-nitrate-cane-sugar-agar (medium given on p. 124 with 40 c.c. soil extract and 10 g. cane-sugar); (4) algae-extract-glucose-agar. For the last, about 100-120 g. of moist algal material from a number of cultures was autoclaved for 20 min. at 10 lb. pressure with 500 c.c. of distilled water, the extract being filtered and sterilized. The medium was as follows: algae extract 200 c.c.; glucose 1 g.;  $K_2HPO_4$  0.2 g.; distilled water to make 500 c.c. Several drops of a suspension of the supposedly pure algal growth were inoculated into the different liquid media, which were then incubated in the dark at 30° C for 4 weeks, the presence or absence of turbidity in the media being taken as an index of the presence or absence of bacteria. Plates were also made with the agar media, but no bacterial colony appeared in any of them. Similar tests were made on suspensions of algal material crushed under water with a glass rod.

Since the value of this investigation depends entirely on the complete elimination of bacteria, a number of cultures were subjected to a critical

examination by Dr Hugh Nicol (Department of Bacteriology, Rothamsted). I take this opportunity to tender him my best thanks for his valuable help. In the report with which he furnished me, he states that he is satisfied, after making diverse tests, that my cultures are free from bacteria.

Stock cultures of the algae used in the subsequent investigation were kept growing in 250 c.c. Erlenmeyer flasks with 100 c.c. of the liquid medium (p. 124) containing 1 % glucose or cane-sugar, so that bacterial contamination would readily be recognized. Subcultures into a fresh medium were made from time to time, but before each transfer the parent culture was thoroughly tested in order to ensure that it had not become contaminated.

#### THE NITROGEN-FIXING CAPACITY OF THE ALGAE

Pyrex Erlenmeyer flasks of 250 c.c. capacity, containing 100 c.c. of liquid medium and plugged with cotton-wool, were sterilized by autoclaving for 20 min. at 20 lb. pressure. The flasks were then allowed to stand for at least 3 days before being inoculated with a suspension of the alga in water. As a rule four replicates were made for each treatment, but in a few experiments the number had to be reduced to three. The parent cultures were grown in mineral salt solution containing 1 % cane-sugar (p. 124) and were 25-30 days old; the age and previous history of these cultures were kept as similar as possible. An attempt was made to test the uniformity of the suspension used by determining the total length of the algal filaments in equal volumes of the suspension. It was found that there was a reasonable degree of uniformity between the different samples taken from one inoculum, but that there was considerable variation in mean fragment length between the inocula prepared on different occasions. This means that in different experiments flasks will have been inoculated with different amounts of the inoculum. Since, however, conclusions are drawn only from individual experiments, and no comparison is made between the results of separate experiments this is immaterial. The slightly varying amounts of inoculum in one and the same experiment will probably have some effect on the initial growth, but it does not seem likely that the ultimate growth will be appreciably affected, since the latter is usually determined essentially by the supply of food material, the accumulation of metabolic products and environmental conditions.

Some experiments were carried out in a greenhouse, others in specially constructed culture chambers in which both temperature and light intensity were controlled. In the greenhouse these two factors varied, but

since all the cultures of a given experiment, and of all the experiments performed simultaneously, were subjected to the same variations, their effect on the ultimate results will have been the same. Comparisons are, however, only valid if made between results obtained in the same or in simultaneous experiments. The temperature of the greenhouse varied between 65 and 80° F in winter and between 70 and 100° F in summer. Sunlight was supplemented by six 150 W lamps arranged in a row at a distance of 2½ ft. from one another and at a height of 1 ft. above the culture flasks. Such illumination was continuous during winter, but confined to night time during summer. The flasks were grouped in random positions on the bench.

Two culture chambers were used, the smaller 18 × 15 in. and the larger 36 × 16 in. The actual chamber (*A* in fig. 1) containing the cultures, 8 in. deep, was surrounded on the bottom and sides by a water-jacket (*B*) heated electrically by evenly distributed heating elements in the compartment *C*; the sides were provided with adjustable ventilators penetrating the water-jacket. The temperature in *A* was regulated by a Hearson capsule *E*, and was maintained at  $35 \pm 1^\circ \text{C}$ . The top of chamber *A* was covered by an easily removable ¾ in. plate glass, *F*. The flasks in *A* were continuously illuminated by eight 25 W lamps in the larger and four 40 W lamps in the smaller apparatus. The lamps were carried on a sheet steel lamp-panel, *D*, supported only at the ends so as to leave the front and back open for manipulation; the panel and its supports were white enamelled on the inside and so acted as reflectors securing more even illumination. All the cultures in a given experiment were grown in the same chamber, and the positions of the flasks were frequently interchanged in order to eliminate as far as possible the effect of any local variation of light intensity. The chambers were screened to shut out daylight and possible illumination from other sources. Special tests showed that an adequate supply of  $\text{CO}_2$  was maintained within the chambers.

The nitrogen-free medium generally used and referred to below as medium I was that described on p. 124, with the omission of potassium nitrate. In certain experiments the following nitrogen-free medium (medium II) was used:  $\text{K}_2\text{HPO}_4$  0.5 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g.;  $\text{CaSO}_4$  0.1 g.;  $\text{FePO}_4$  0.1 g.;  $\text{Ca}_3(\text{PO}_4)_2$  1.0 g.;  $\text{FeCl}_3$  (1 %) 2 drops; water (pyrex distilled) 1000 c.c. This is adapted from one used by Thornton (1930) for growing lucerne and has been employed extensively in India for growing the algae of rice fields.

At the conclusion of an experiment the entire contents of the culture vessel (medium + alga) were poured into a Kjeldahl flask, any growth

adhering to the side of the vessel being washed out with pure concentrated  $\text{H}_2\text{SO}_4$ . Total nitrogen was estimated by the Kjeldahl-Gunning method. Digestion and distillation were carried out in the usual way, the ammonia evolved being absorbed in  $\text{N}/10 \text{ H}_2\text{SO}_4$  and estimated by back titration with  $\text{N}/10 \text{ NaOH}$ , using methyl red as an indicator. For the determination of

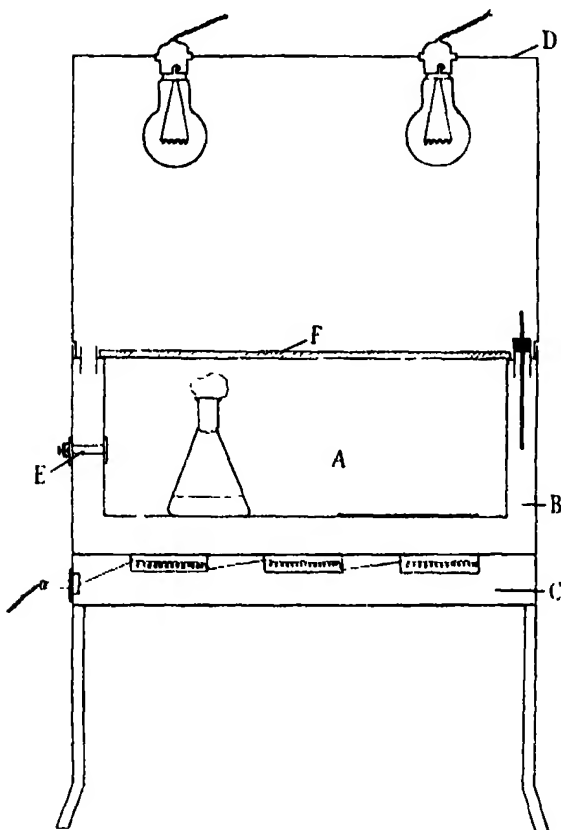


FIG. 1

small amounts of nitrogen, e.g. that in the external medium,  $\text{N}/50$  acid and alkali were used, but the acid was boiled so as to drive off  $\text{CO}_2$  before titration. Before analysing cultures grown on a medium originally containing nitrate, a few drops of the culture liquid were tested with diphenylamine. Such tests for nitrate were always negative. Blank estimations on the uninoculated medium (N-free) and the reagents were made from time to time. The value never exceeded 0.1 c.c. of  $\text{N}/10 \text{ H}_2\text{SO}_4$  and was subtracted from the result of analysis.

(a) *Anabaena variabilis* (Table I)

One series of experiments with this alga was carried out in the culture chamber (A) and the other in the greenhouse (B). In the former series the following media were used: (1) medium I (p. 128), N-free; (2) medium I + soil extract; (3) medium I + nitrate; (4) medium I + nitrate + soil extract. In the greenhouse series the same media were used, except that in (1) medium II (p. 128) was substituted.

TABLE I. NITROGEN FIXATION BY *ANABAENA VARIABILIS*

Nitrogen in mg. per 100 c.c. of the medium. Period of incubation—60 days

Media	N at start	Culture chamber (A)			Greenhouse (B)		
		N after the experiment	N fixed	Average of the series	N after the experiment	N fixed	Average of the series
1. N-free (medium I in A; medium II in B)	—	2.8	2.8	2.6	3.8	3.8	4.1
		2.4	2.4		4.3	4.3	
		2.7	2.7		4.3	4.3	
		2.5	2.5		4.2	4.2	
2. N-free + soil extract	0.3	5.4	5.1	4.7	4.0	3.7	3.9
		5.0	4.7		4.2	3.9	
		4.3	4.0		4.5	4.2	
		5.4	5.1		4.1	3.8	
3. N-free + nitrate	2.0	3.2	0.3	0.7	3.2	0.3	0.4
		3.5	0.6		3.4	0.5	
		4.1	1.2		3.4	0.5	
		3.5	0.6		—	—	
4. N-free + nitrate + soil extract	3.2	9.1	5.9	5.7	8.6	5.4	4.1
		8.6	5.4		7.8	4.6	
		8.5	5.3		7.5	4.3	
		9.6	6.4		5.4	2.2	

In the first series the growth was at first rapid and no distinction could be drawn between the cultures with and without nitrate. After some days, however, all the nitrate cultures, especially those containing soil extract, became pale or nearly colourless, while those without nitrate remained as green as before. In both there was little submerged growth, the major portion floating at the surface, and there was a tendency for the alga to grow above the level of the fluid along the sides of the flask. By the end of the second week only the cultures without nitrate, but containing soil extract, remained green, although occasional green patches became noticeable on the nearly colourless growth of the nitrate + soil-extract



cultures. These patches gradually increased in extent, and by the end of the third week the alga in these particular cultures again became green and showed renewed growth. No other marked change in the condition of the cultures was noticed during the remainder of the experiment.

The growth in the greenhouse series was also rapid in the beginning, though entirely submerged. Here, too, the nitrate cultures became pale green after some time. The cultures with nitrate + soil extract did not remain in this condition for long, but, like the corresponding cultures in the chamber, again became deep green and showed renewed growth. The cultures grown in medium II, without soil extract, remained deep green throughout, thus contrasting with the corresponding cultures in the first series, which were grown in medium I. In the greenhouse only those cultures grown with nitrate and without soil extract remained pale up to the end.

(b) *Anabaena gelatinosa* (Table II)

The experiments with this alga were performed only under greenhouse conditions, using the same media as in the second series with *A. variabilis*. While the latter alga in the early stages of growth forms a number of minute colonies, *A. gelatinosa* quickly forms a continuous stratum over the bottom, gradually spreading from here until it finally covers the entire

TABLE II. NITROGEN FIXATION BY *ANABAENA GELATINOSA*

Nitrogen in mg./100 c.c. of the medium.  
Period of incubation — 60 days (greenhouse cultures)

Media	N at start	N after the experiment	N fixed	Average of the series
1. N-free	—	3.0	3.0	3.5
		3.7	3.7	
		3.2	3.2	
		4.0	4.0	
2. N-free + soil extract	0.3	4.5	4.2	4.4
		4.4	4.1	
		5.0	4.7	
		4.8	4.5	
3. N-free + nitrate	2.9	3.2	0.3	0.4
		3.4	0.5	
		3.2	0.3	
		—	—	
4. N-free + nitrate + soil extract	3.2	7.2	4.0	3.9
		7.0	3.8	
		7.0	3.8	
		7.3	4.1	

submerged surface of the flask. In older cultures the stratum becomes detached and remains suspended in the solution as small flakes.

The growth of *A. gelatinosa* in nitrogen-free solution was nearly as rapid as in the presence of nitrate. In the early stages the nitrate cultures were of a deeper colour than those growing without nitrate, but this condition was reversed in the later stages. Cultures grown with nitrate + soil extract showed the same features as those of *A. variabilis*; they were green at first, then became almost colourless and subsequently again became green.

(c) *Anabaena naviculoides* and *Phormidium foveolarum*

These were not examined in such great detail, a little growth from a liquid culture of each being introduced into medium II and into one made up of medium I with soil extract + nitrate. The cultures were kept in the greenhouse for 60 days. *Anabaena naviculoides* grew well in both solutions, but *Phormidium foveolarum* made absolutely no growth in the nitrogen-free solution, although in nitrate + soil extract the growth was quite abundant.

*Nitrogen fixation by Anabaena naviculoides and  
Phormidium foveolarum*

(Nitrogen fixed in mg./100 c.c. of the medium)

	N-free medium	Nitrate + soil extract
<i>Anabaena</i>	2.7	3.8
<i>naviculoides</i>	2.6	3.5
<i>Phormidium</i>	No growth	Nil
<i>foveolarum</i>		0.2

The results of the analyses of the different experiments are considered below. In order to determine the distribution of the nitrogenous substances formed, between the alga and the surrounding medium, some of the cultures in the above experiments were filtered before analysis, and the clear filtrate and the algal growth analysed separately. The results in Table III show that a considerable part of the nitrogen is present in the external medium. A portion of the latter was distilled with MgO, but no ammonia was formed. Evidently all the nitrogen was present in an organic form, and was completely precipitated by basic lead acetate.

In those and subsequent experiments the observed increase in nitrogen might be due to absorption of a certain amount of ammonia or of oxides of nitrogen from the air, but this is disproved by the fact that the uninoculated control flasks (N-free), exposed side by side with the cultures, did not show any increase in nitrogen. An experiment was, moreover, performed in which the culture flasks were constantly aerated with air freed from

TABLE III. NITROGEN IN THE ALGAL BODIES AND  
IN THE EXTERNAL MEDIUM AS MG. PER FLASK

Media	Total N	N in algal bodies	N in external medium	N in external medium as % of total N
<i>Anabaena variabilis</i>				
N-free	2.8	1.8	0.98	35
N-free + soil extract	5.4	3.0	2.4	44
N-free + soil extract + nitrate	9.6	6.9	2.7	28
<i>Anabaena gelatinosa</i>				
N-free	4.0	2.7	1.3	32
N-free + soil extract	4.4	3.0	1.4	32
N-free + soil extract + nitrate	7.3	5.9	1.4	19

ammonia and nitrous gases; the amount of nitrogen fixed did not differ materially from that fixed in non-aerated cultures.

The abundant growth of all three species of *Anabaena* in the nitrogen-free solution indicates their capacity to assimilate elementary nitrogen. This was confirmed by analysis of the cultures which showed increases in nitrogen far beyond any which could be attributed to experimental error. *Phormidium foveolarum*, on the other hand, showed no growth whatever in the nitrogen-free solution, and although growth was considerable in nitrate + soil extract, the total nitrogen content of the cultures at the end of the experiment was the same as at the beginning. A second experiment gave the same result. Evidently this alga is quite unable to fix atmospheric nitrogen.

It is remarkable that in the presence of nitrate (without soil extract) there was scarcely any fixation of nitrogen, either by *Anabaena variabilis* or by *A. gelatinosa*, although the growth was good. An analysis of the medium at the end of the experiment showed a complete absence of nitrate, so that the organisms in these experiments grew only at the expense of the combined nitrogen present in the culture solution. It thus appears that, as long as suitable nitrogen compounds are present, the algae in question assimilate these latter and do not fix nitrogen. In this respect their behaviour is similar to that of *Azotobacter*, which, as Bonazzi (1921) has shown, has recourse to nitrogen fixation only if starved of nitrogenous compounds.

The addition of soil extract stimulates the fixation of nitrogen in certain instances. Thus, in the experiments with *A. variabilis* using medium I

(cf. Table I), the alga fixed much more nitrogen when a little soil extract was added to the solution; in other experiments (B, 1 and 2) in which medium II was used, this effect was not observed. The stimulating effect of soil extract is also clearly seen in the nitrate cultures (3 and 4). As already noted, these in the absence of soil extract (3) exhibit scarcely any fixation, but there is a considerable increase in the amount of nitrogen fixed when a little soil extract is added (4), although here, too, there is no nitrate left in the medium at the end of the experiment. The organisms in such cultures probably grow initially at the expense of the nitrate, and it is only after the supply of the latter is exhausted that the more complex process of nitrogen fixation sets in.

The influence of soil extract in stimulating nitrogen fixation may be due to the additional nutrients supplied or to the addition of growth-promoting substances. It has already been pointed out that the amount of nitrogen fixed by *A. variabilis* and by *A. gelatinosa* in medium I+soil extract is essentially the same as that fixed in medium II without soil extract (cf. Tables I and II). Medium II is, however, richer in nutrients than medium I. This would, therefore, suggest that the effect of addition of soil extract is to supply additional nutrients. It is difficult, however, to accept this as the only factor involved, since the quantity of soil extract added (4 c.c. in 100 c.c. of the medium) is too small to contribute any appreciable amount of nutrients. Soil infusion also has a stimulating effect on the fixation of nitrogen by *Azotobacter*, and various hypotheses have been put forward to explain its action. Remy and Rosing (1911) and Carsten Olsen (1930) attribute the action to the iron present, while Allen (1919) supposes that the colloids of the humus prevent complete precipitation of the phosphates and thus facilitate phosphatic nutrition of the *Azotobacter*. The beneficial effect of humus has also been ascribed to its inorganic constituents, particularly aluminium and silicic acid.

It remains to consider the role of bacteria in the process of nitrogen fixation in the rice fields. It is possible that the algae, while fixing nitrogen themselves, might simultaneously afford conditions favourable for the activities of nitrogen-fixing bacteria. Such relations have frequently been discussed (see the summary in Bristol-Roach 1927) before it was established that certain algae could themselves fix elementary nitrogen. The algae have been thought to stimulate the fixation of nitrogen by bacteria either by utilizing the nitrogenous products of the latter and thus creating conditions favourable for their continued activity or by furnishing synthesized carbohydrate materials serving as a source of energy. The first hypothesis does not, however, seem very probable, since algae grow much more

slowly than bacteria or fungi, so that nitrogenous products, if any, liberated in the soil might be quickly acted upon by other types of bacteria and fungi before algae could avail themselves of them.

On the other hand bacteria (not necessarily nitrogen-fixing species) might absorb materials produced by the algae so that an association of the latter with bacteria might stimulate nitrogen fixation by the algae. In order to investigate this point two similar cultures of *Anabaena variabilis*, one devoid of bacteria and the other contaminated with them, were set up under greenhouse conditions and the amounts of nitrogen fixed in two months determined. The average of four experiments gave for the pure culture 4.2 mg. and for the impure 3.7 mg. of nitrogen, which does not favour the view that the presence of bacteria has any effect on the fixation of nitrogen.

As regards the second hypothesis mentioned above it seems possible that the carbohydrate materials synthesized by algae may serve, either directly or after transformation into simpler substances, as sources of energy for nitrogen-fixing bacteria. An experiment was carried out to investigate such availability, using *Azotobacter chroococcum*, an organism present in abundance in Faridpur soil (De and Pain 1936). Three sets of cultures were undertaken—the first with the alga alone, the second with alga + *Azotobacter*, and the third with *Azotobacter* alone. Medium I was employed, but

TABLE IV. INFLUENCE OF *AZOTOBACTER* ON THE  
FIXATION OF NITROGEN BY ALGAE

Nitrogen in mg./100 c.c. of the medium (the numbers in parentheses are averages)

<i>Anabaena variabilis</i>		<i>Anabaena gelatinosa</i>	
Alga	Alga + <i>Azotobacter</i>	Alga	Alga + <i>Azotobacter</i>
3.4	3.7	3.5	3.0
4.0 (3.7)	3.8 (3.8)	2.9 (3.2)	3.0 (3.1)
3.7	3.8	3.2	2.9
3.5	4.0	3.0	3.4

in order to encourage the growth of *Azotobacter*, the amount of phosphate was increased to 0.5 g./l.; soil extract was added in the usual proportion. The second and third cultures were inoculated, both at the beginning of the experiment and again after a period of 1 month, with a suspension in water of a 4-day old culture of *Azotobacter* grown on Ashby's agar. The cultures remained in the culture chamber for 2 months, after which they were analysed for total nitrogen. There was no fixation of nitrogen in the cultures containing *Azotobacter* only.

The amount of nitrogen fixed by the mixed cultures did not differ from that in the cultures containing the alga only. Microscopic examination of the former at the conclusion of the experiment showed scarcely any *Azotobacter*, indicating that the conditions were not favourable for its development. These results may be due to a variety of causes, but they do not favour the view that *Azotobacter* is able to use directly the carbohydrate materials synthesized by the algae in question.

In the next place an endeavour was made to ascertain whether dead algal bodies, undergoing decomposition in the soil, could provide materials suitable to act as a source of energy. In this experiment soil was used as the medium in order to facilitate the growth of different kinds of micro-organisms present in it. A mixture of 15 g. of Faridpur soil and 100 c.c. of distilled water was placed in each of twelve Erlenmeyer flasks plugged with cotton-wool. Six of the flasks were sterilized by autoclaving at 20 lb. pressure for half an hour on two consecutive days, and then inoculated with a suspension in water of bacteria-free *Anabaena variabilis*. The whole series of flasks was then removed to the culture chamber and kept there for 2 months during which abundant growth of algae took place in all. At the end of this period, the contents of six flasks—three with sterilized and three with unsterilized soil—were analysed for total nitrogen. The remaining six flasks were sterilized, which of course killed the contained algae, inoculated with a little suspension of Faridpur soil and incubated for a month at 30° C in the dark. At the end of the month the total nitrogen in these flasks was estimated.

The results of this experiment are given in Table V. Series (1) and (3) show fixation of nitrogen by the algae, while series (2) and (4) show the effect of the decomposition of the algae grown in (1) and (3) respectively on the total nitrogen content of the soil. These results bring out several facts. Series (1) shows that algae can grow and fix nitrogen *in the soil*, independently of the presence of bacteria or fungi. It is of interest that in unsterilized soil (series 3), where perhaps a number of algae worked in conjunction with bacteria or fungi, as much nitrogen was fixed as in the sterilized soil where only one species of alga and no bacteria or fungi were concerned. This provides additional support for the view already expressed that the activity of the algae is not affected by the presence of other organisms. A comparison of series (1) and (2), as well as of (3) and (4), demonstrates that the presence of decomposing algal bodies does not result in any appreciable increase in the total nitrogen content of the soil, which tends to suggest that such products of decomposition do not serve as a source of energy for the nitrogen-fixing bacteria. This conclusion is,

TABLE V. INFLUENCE OF THE PRESENCE OF DECOMPOSING ALGAE IN THE SOIL ON THE FIXATION OF NITROGEN

(Nitrogen in mg. in 15 g. of soil; the numbers in parentheses are averages)

Series	N at start	N after the experiment	N fixed
(1) Sterilized soil + alga	8.3	14.1	5.8
		13.6	5.3 (5.6)
		14.1	5.8
(2) Same cultures sterilized after 2 months and inoculated with soil suspension	8.3	13.8	5.5
		13.8	5.5 (5.6)
		14.1	5.8
(3) Unsterilized soil	8.3	13.8	5.5
		13.8	5.5 (5.6)
		13.9	5.6
(4) Same cultures sterilized after 2 months and inoculated with soil suspension	8.3	13.6	5.3
		14.1	5.8 (5.5)
		13.8	5.5

however, open to criticism on the ground that the amount of energy-giving material available is too small to allow of the fixation of any measurable amount of additional nitrogen. However that may be, the results show that bacteria in these soils play a relatively unimportant role in the fixation of nitrogen.

A consideration of the C : N ratio of the algae involved demonstrates that they do not afford material suitable to serve as a source of energy for nitrogen-fixing bacteria. The C : N ratio of *Anabaena variabilis* is 10 : 1 (C % 30.15, N % 3.0). Winogradsky and Ziemiecka (1928) inoculated soil rich in *Azotobacter* to a medium containing mannitol and varying doses of nitrate and found that the development of *Azotobacter* became less and less vigorous with increasing amount of nitrate and that it stopped completely when the C : N ratio reached 100 C : 0.4 N. Waksman and Tenney (1927) have shown that plant materials containing more than 1.7 % of nitrogen decompose in the soil with immediate production of ammonia. A similar observation has been made by Jensen (1929), who found that, when organic materials with a C : N ratio less than 26 : 1 decomposed in an alkaline soil (pH 7.42), a part of the nitrogen readily became available.

In view of these conclusions it may probably be assumed that decomposition of algae in the soil will be attended by an immediate production of ammonia. It is well known that the addition of ammonium sulphate, of nitrate and of materials rich in nitrogen to the soil tends to check the development of *Azotobacter*. Thus, Ziemiecka (1932), who determined the

numbers of *Azotobacter* in the soils of the classical plots at Rothamsted, found that the highest number of colonies was afforded by the plots receiving complete minerals but no nitrogen, while the plots receiving nitrogen showed no or few colonies of *Azotobacter*. It was suggested that this was due to competition with other organisms the growth of which was stimulated by added nitrogen compounds. It, therefore, follows that the decomposition of algae in the soil, far from encouraging the growth of *Azotobacter*, will have a depressing effect upon it.

The above considerations indicate that it is legitimate to conclude that the fixation of nitrogen in the soil of the rice fields is brought about mainly by algae.

In conclusion the author wishes to express his gratitude to Professor F. E. Fritsch, under whose supervision the present investigation was carried out, to Dr F. M. Haines for valuable suggestions and continuous help, and to the Imperial Council of Agricultural Research in India for enabling the author to visit England in order to carry out the investigation.

#### SUMMARY

The investigation deals with nitrogen fixation by certain blue-green algae isolated from the soil of an Indian rice field. By repeated subculturing on sterilized silica gel plates three species of *Anabaena* and *Phormidium foveolarum* were obtained in pure bacteria-free cultures. Cultures from inocula of the species of *Anabaena* after growing for some weeks in nitrogen-free solutions exhibited considerable increase in nitrogen, and such nitrogen fixation was stimulated by the addition of small amounts of soil extract. *Phormidium foveolarum*, on the other hand, afforded no evidence of nitrogen fixation. A considerable part of the nitrogen fixed is found in the external medium in an organic form. Evidence was obtained that algae are the main agents of nitrogen fixation in the rice fields, and that the part played by bacteria is relatively unimportant and possibly nil.

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# Synthetic oestrogenic compounds related to stilbene and diphenylethane. Part I

By E. C. DODDS, L. GOLBERG, W. LAWSON  
AND SIR ROBERT ROBINSON, F.R.S.

*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School,  
London, and Dyson Perrins Laboratory, Oxford University*

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## INTRODUCTION

The present investigation is a natural combination of two converging lines of development.

In the first place the oestrogenic activity of some quite simple compounds was noted and efforts were made to modify the structures in the hope of increasing their potency. Among these substances the following may be mentioned:

1-keto-1:2:3:4-Tetrahydrophenanthrene (Cook, Dodds, Hewett and Lawson 1934);

4:4'-Dihydroxydiphenyl;

*p-n*-Propylphenol;

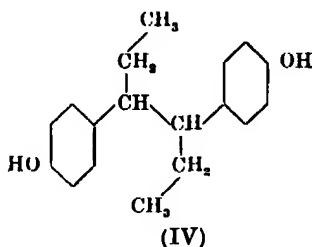
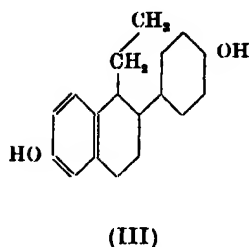
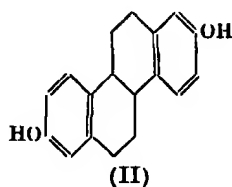
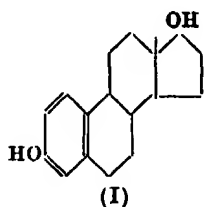
*p*-Hydroxybutyrophenone (Dodds and Lawson 1938);

4:4'-Dihydroxydiphenylethane and 4:4'-dihydroxystilbene (Dodds and Lawson 1937; Dodds, Fitzgerald and Lawson 1937).

Attention from this point of view was concentrated on the diphenylethane and stilbene series following the discovery that the product of demethylation of anethole contains a potent substance which is not *p*-propenylphenol (Dodds and Lawson 1937); it was obvious that this substance might be a diphenylethane derivative.

Secondly, in view of the difficulty presented by the total synthesis of the naturally occurring oestrogenic hormones, attempts were made to prepare more accessible substances the molecules of which would be similar to oestradiol (I) in shape, weight, and in the situation of the hydroxyl groups. Taking cognizance of the known weak but definite oestrogenic activity of 4:4'-dihydroxydiphenyl it appeared that both hydroxyls might well be

phenolic and the types II, III and IV were indicated as worthy of detailed study. The two approaches meet in the compound IV:



The dimethyl ether of II (*trans*-fusion of the tetralin nuclei) had already been prepared (Ramago and Robinson 1933, 1935) and on demethylation by means of hydriodic acid it affords the *dihydroxyhexahydrochrysene* which was found to possess oestrogenic properties (fully oestrogenic in doses of 0.001 g. to ovariectomized rats). A specimen of an oily product thought to be IV (see, however, p. 144) had an oestrogenic potency of about the same order.

As the result of a very recent observation that the active compound produced in the course of the demethylation of anethole (Campbell, Dodds and Lawson 1938) actually corresponds to the structure IV, the earlier results are being reviewed and extended. We are attempting the resolution of the substance in order to determine which of the two known isomerides (see p. 154) corresponds to the *racemic* and which to the *meso* configuration.

During the period of the present research we were impressed by the fact that 4:4'-dihydroxystilbene is more active than 4:4'-dihydroxydiphenylethane and hence concentrated on the examination of C-alkylated derivatives of the former substance which may be termed "stilboestrol".

The method of synthesis used was selected because it facilitated the variation of the substituents. Deoxyanisoin was alkylated (*R*) and then brought into reaction with a Grignard agent (*R'*Mg hal.). The resulting carbinol (V) was then dehydrated with formation of the dialkylstilboestrol dimethyl ether (VI). Demethylation by acids or aluminium halides failed

but could be effected by means of hot alcoholic potassium hydroxide, the reaction being carried out in an autoclave.

Mono-alkylated stilboestrols were similarly prepared, omitting the stage of alkylation of deoxyanisoin:

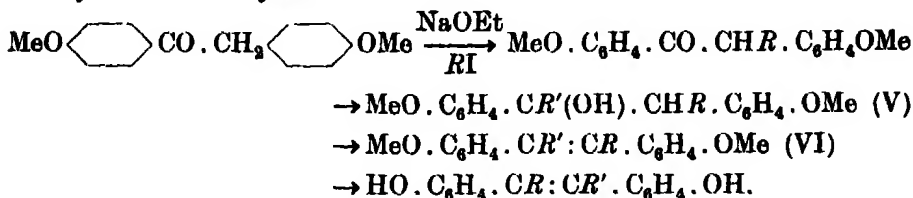
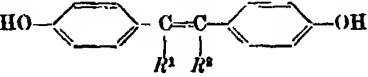


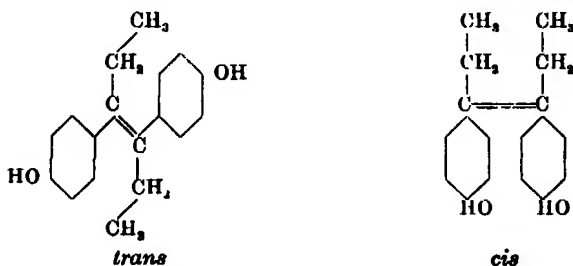
TABLE I

		Dose in mg.	% positive	Rat units per g. estimated
$R^1$	$R^2$			
H	H	5	80	140
		10	100	
H	$\text{C}_2\text{H}_5$	0.1	50	5,000
H	$\text{C}_4\text{H}_9$	0.1	100	10,000
$\text{CH}_3$	$\text{CH}_3$	0.02	80	40,000
		0.03	100	
$\text{CH}_3$	$\text{C}_2\text{H}_5$	0.0005	30	1,000,000
		0.001	100	
$\text{C}_2\text{H}_5$	$\text{C}_2\text{H}_5$	0.0003	80	3,000,000
		0.0004	100	
$\text{C}_2\text{H}_5$	$n\text{-C}_3\text{H}_7$	0.001	Trace	300,000
		0.01	100	
$\text{C}_2\text{H}_5$	$\text{---CH=CH---CH}_3$	0.001	60	400,000
		0.005	100	
$\text{---CH=CH---CH}_3$	$\text{---CH=CH---CH}_3$	0.01	30	20,000
		0.1	100	
$n\text{-C}_3\text{H}_7$	$n\text{-C}_3\text{H}_7$	0.01	75	50,000
		0.1	100	
$iso\text{-C}_3\text{H}_7$	$iso\text{-C}_3\text{H}_7$	0.025	60	20,000
		0.1	100	
$n\text{-C}_4\text{H}_9$	$n\text{-C}_4\text{H}_9$	0.01	Nil	5,000
		0.1	40	
Monohydroxydiethylstilbene		0.1	Trace	10
		1.0	100	

The results of biological tests (Table I) show that a remarkable peak in oestrogenic activity is found in diethylstilboestrol, and it is at least a

noteworthy coincidence that the centrally situated chain of six carbon atoms corresponds exactly with the oestradiol structure (I).

We were able to isolate an isomeride of diethylstilboestrol from the mother liquors of the last stage of the preparation and this substance,  $\psi$ -diethylstilboestrol, is probably the *cis*-stilbene derivative. As shown in Table II, which includes some miscellaneous results,  $\psi$ -diethylstilboestrol is a weaker oestrogenic agent than diethylstilboestrol itself. By all analogies this latter substance should be the *trans*-derivative which, as indicated by the models, is clearly the more closely related stereochemically to oestradiol:



We may anticipate that the further study of the stereoisomeric dihydroxy-diethyldiphenylmethanes will throw further light on the validity of this argument to which we attach very great importance.

TABLE II

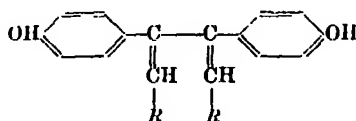
Substance	Dose in mg.	% positive
4:4'-Dihydroxy- $\alpha$ -benzylidenediphenylmethane	0.1	100
$\psi$ -Diethylstilboestrol	(0.5 $\gamma$ )	25
	(1.0 $\gamma$ )	100
1-Ethyl-2 ( <i>p</i> -hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene	1.0	20
	10.0	100
	0.01	20
	0.1	100
4:4'- $\alpha\beta$ -Tetrahydroxydimethyldiphenylethane	100.0	100
4:4'- $\alpha\beta$ -Tetrahydroxydiethyldiphenylethane	0.1	100
	0.01	80
4:4'- $\alpha\beta$ -Tetrahydroxydipropyldiphenylethane	10.0	100

Table II also includes a naphthalene derivative obtained in the course of attempts to obtain substances of type III. In the course of our work we

had occasion to prepare pinacols of *p*-hydroxyacylbenzenes; these substances are  $\alpha\beta$ -dihydroxy-derivatives of 4:4'-dialkyldiphenylmethanes and they exhibit oestrogenic properties (see Table II).

On dehydration by means of a boiling mixture of acetyl chloride and acetic anhydride these pinacols afford substituted 4:4'-dihydroxydiphenylbutadienes which are powerful oestrogenic agents. As shown in Table III the maximum activity is again found when the central chain comprises six carbon atoms. 4:4'-Dihydroxy- $\gamma$ - $\delta$ -diphenyl- $\beta$ : $\delta$ -hexadiene, later termed the "diene", is, in fact, about as potent as diethylstilboestrol and, as mentioned below, may be superior to it in some respects.

TABLE III



	Dose in mg.	% positive
4:4'-Dihydroxy- $\beta$ : $\gamma$ -diphenylbutadiene ( $R = \text{H}$ )	10 1	100 Trace
4:4'-Dihydroxy- $\gamma$ : $\delta$ -diphenyl- $\beta$ : $\delta$ -hexadiene ( $R = \text{CH}_3$ )	0.0005 0.0004	100 70
4:4'-Dihydroxy- $\delta$ : $\epsilon$ -diphenyl- $\gamma$ : $\epsilon$ -octadiene ( $R = \text{C}_2\text{H}_5$ )	0.01 0.002	100 Nil
$\beta$ : $\gamma$ -Di (4-hydroxyphenyl)- $\alpha$ : $\delta$ -diphenylbutadiene ( $R = \text{C}_6\text{H}_5$ )	10	Nil

On catalytic reduction this diene gives a good yield of a tetrahydro-derivative (formula IV), m.p.  $184^\circ$ , which substance has been identified by Campbell, Dodds and Lawson (1938) with the highly oestrogenic compound separated by them from the products of the demethylation of anethole. Catalytic reduction of  $\psi$ -diethylstilboestrol gives the same substance in poor yield; the main product from diethylstilboestrol is a more fusible isomeride.

#### THE BIOLOGICAL ACTIVITY OF THE SYNTHETIC SUBSTANCES

In the course of the work already described biological control was established by determining the amount of substance required to produce oestrus when injected subcutaneously into rats. The method employed was that of Allan, Dickens and Dodds (1930).



In earlier experiments it has been reported that a number of substituted dibenzanthracenediols possess all the known activity of the naturally occurring hormone. Thus Wolfe (1936) has shown that the di-*n*-propyl substituent is capable of preventing the castration changes in the anterior lobe of the pituitary of ovariectomized animals, whilst Hemmingsen and Krarup (1937) demonstrated that the same compound will restore the mating instinct to the ovariectomized rat. It is also capable of sensitizing the action of the uterus to progesterone.

Very careful biological experiments were performed with the highly active substances obtained as a result of the present study. In the first instance an attempt to compare the activities of oestrone, oestradiol diethylstilboestrol and dihydroxydiphenylhexadiene was made. Groups of between forty-four and fifty-seven rats were employed in these experiments which are shown in Table IV. Considerable difficulty arose in the evaluation of the results. For example, it is well known that the smear may show complete oestrus, or a smear with just a few nucleated cells, which is known as *E*-. Again, with the presence of more nucleated cells this may be termed very advanced pro-oestrus. In the table, evaluation on these lines will be found. From a study of the results it would appear that diethylstilboestrol is roughly  $2\frac{1}{2}$  times more potent than oestrone, but not so active as oestradiol. The diene compound appears to possess an activity just a little less than diethylstilboestrol.

TABLE IV

Substance	Dose	No. of rats	% positive		
			<i>E</i>	<i>E</i> -	<i>P</i> <sup>a</sup>
Oestrone	0.6	57	21.0	8.8	33.4
	0.6	57	15.8	7.0	8.8
	0.7	56	50.0	8.9	19.6
	0.8	57	88.0	7.0	3.5
Oestradiol	0.1	56	17.8	—	39.3
	0.2	54	94.5	1.85	1.85
Diethylstilboestrol	0.25	56	35.6	8.9	25.7
	0.30	55	52.8	3.6	25.5
	0.35	51	98.0	—	—
Dihydroxydiphenylhexadiene	0.3	50	18.0	12.0	22.0
	0.35	49	20.4	—	36.8
	0.4	50	86.0	8.0	—

*Action by mouth.* The activity of the diethylstilboestrol and the diene were examined by mouth. Doses varying from 0.1 to 5γ were dissolved in 3 c.c. sesame oil and administered in 6 doses of 0.5 c.c. morning and evening

for 3 days. Each dose was dropped into the animal's mouth by means of a pipette. Negative results were obtained with doses of 0.1 and 0.5 $\gamma$  in the case of diethylstilboestrol, whilst 1.0 $\gamma$  gave a 70 % response and 5.0 $\gamma$  produces oestrus in 100 % of the rats. 100 % oestrus was produced with 3 $\gamma$  of the diene.

It has been known for some considerable time that large doses of oestrone are required to confer on ovariectomized female rats the mating instinct. Experiments have shown that with quantities in excess of 200 $\gamma$  injected subcutaneously, vaginal plugs will be seen in some of the treated animals. In the case of diethylstilboestrol injected subcutaneously, plugs were found with quantities from 50 to 400 $\gamma$ . It was found impossible, however, to give any indication as to the quantitative relationship between the number of vaginal plugs and the amount of diethylstilboestrol administered, but this is also the case with the naturally occurring hormone.

The general biological action of diethylstilboestrol has been described (Dodds, Lawson and Noble 1938). It has been shown that the compound will produce full vaginal oestrus in ovariectomized rats, will sensitize the rabbit uterus to the action of progesterone and also confer the mating instinct on the ovariectomized animal. It is difficult to secure an accurate comparison as to the quantity required relative to oestrone, owing to the differences in rates of absorption and excretion. The action of the compound on the anterior lobe of the pituitary appears to be similar in every respect to that of oestrone. Thus it has been shown (Noble 1938*a*) that both diethylstilboestrol and the diene will inhibit the growth of immature animals when implanted under the skin. In this respect the diene appears to be more potent than the diethylstilboestrol, probably due to the fact that it is more slowly absorbed. A further observation of the action of diethylstilboestrol on the anterior lobe of the pituitary has been reported by Noble (1938*b*). It was found that the testes, prostate and seminal vesicles in the male, and ovaries in the female, atrophied rapidly with prolonged injection and there was also a very marked increase in the weight of the adrenals and suprarenals of both sexes. Since an increase in ovarian weight will occur in such treated animals when injected with pregnancy urine extract, it is reasonable to suppose the original atrophy is due to the action of the anterior lobe of the pituitary. Studies of the water balance failed to demonstrate any alteration in the secretion of the diuretic principle of the anterior lobe. An interesting action has also been observed by Parkes, Dodds and Noble (1938). It has been known for some time that oestrone will inhibit the pregestational phase of the uterine oestrus cycle, and that it is possible to interfere with implantation of the fertilized ovum through this mechanism.

Since, however, neither oestrone nor oestradiol are particularly active by mouth, there appeared to be no possibility of a practical outcome of this observation. It was shown, however, that diethylstilboestrol when administered by mouth to animals is capable of inhibiting the progestational phase, and by this means pregnancy may either be prevented through lack of nidation or alternatively may be terminated at a very early stage through absorption of the implanted ovum. Folley has shown (1938) that diethylstilboestrol produces the same effect on the mammary glands and on the composition of milk as oestrone.

The general toxicity of the substances has been investigated, and when administered in large doses, either subcutaneously or by mouth, they appear to be without poisonous properties relative to their biological activity. Thus twelve rats were given 10 mg. each for 20 days, in an experiment to investigate the metabolism of the substance. No toxic effects were observed. It would appear that the above could be used clinically, and at the present time trials are being undertaken. One paper has appeared (Guldberg 1938) describing the treatment of a patient suffering from severe menopausal symptoms as a result of bilateral ovariectomy. The symptoms were controlled in the first instance by subcutaneous injection of diethylstilboestrol dissolved in oil, and later the subject was maintained in good health by oral administration. It is obvious, however, that a very large series of clinical experiments must be performed before the general use of the substance can be recommended.

Although diethylstilboestrol is a powerful oestrogenic agent it appears to be quickly eliminated and the duration of oestrus is relatively brief. In the case of the natural hormones it is known that action may be greatly prolonged by the preparation and use of esters. Table V shows that analogous results may be obtained with esters of diethylstilboestrol, and, of the substances so far examined, the dipropionate gives the best combination of activity and prolonged duration of oestrus.

#### PREPARATION AND DESCRIPTION OF NEW SUBSTANCES

*4-Phenyl-3-anisylhexan-3-ol.* A solution of 4-methoxy- $\alpha$ -ethyldeoxybenzoin (Ney 1888) (17 g.) in dry ether (75 c.c.) was added slowly to a cooled Grignard solution from ethyl bromide (23 g.) and magnesium (5 g.) in ether (125 c.c.). After gently heating on the steam bath for an hour the reaction mixture was decomposed with ice and ammonium chloride, and the ethereal layer dried and evaporated. The residue was a colourless viscous liquid,

b.p. 140–143°/0.3 mm. (15.1 g.) (Found: C, 80.4; H, 8.4.  $C_{18}H_{24}O_2$  requires C, 80.2; H, 8.5 %).

4-Methoxy- $\alpha$ : $\beta$ -diethylstilbene. The above carbinol was dehydrated with phosphorus tribromide in chloroform solution. The product, isolated in the usual manner, was a colourless oil, b.p. 140–144°/0.25 mm. (Found: C, 85.0; H, 8.1.  $C_{18}H_{22}O$  requires C, 85.6; H, 8.3 %).

TABLE V. ACTIVITY OF ESTERS OF DIETHYLSTILBOESTROL

Substance	Dose (in $\gamma$ )	Average duration of oestrus (days)
Diethylstilboestrol	1	4
	5	4
	10	5
Diethylstilboestrol di-acetate	10	21
di-propionate	10	52
di-n-butyrate	10	10
di-iso-butyrate	10	4
di-n-valerate	10	2
di-phenylacetate	10	4
	50	30
di-palmitate	50	2
	100	77*
di-benzoate	10	Nil
	100	77*
di- $\alpha$ -naphthoate	100	3
di- $\beta$ -naphthoate	100	3
di-methyl ether	50	2
	1000	144†
	6600	180

\* All animals in oestrus when killed.

† Two animals in oestrus when killed after 183 days.

4-Hydroxy- $\alpha$ : $\beta$ -diethylstilbene. A mixture of the methyl ether (3.9 g.), potassium hydroxide (8 g.) and alcohol (10 c.c.) was heated to 190° for 20 hr. The clear solution became opalescent on dilution with water and the free hydroxystilbene could be extracted therefrom by ether. The ethereal solution was dried and evaporated and the residue distilled as a colourless oil, b.p. 135–140°/0.15 mm. (Found: C, 85.6; H, 8.1.  $C_{18}H_{20}O$  requires C, 85.7; H, 8.0 %).

3:4-Dianisylhexan-3:4-diol. A solution of anisil (27 g.) in warm benzene (500 c.c.) was added dropwise to a Grignard solution prepared from magnesium (12.2 g.), ethyl bromide (54.5 g.) in dry ether (300 c.c.). The mixture was refluxed on the steam-bath for 2 hr. Ice and ammonium chloride were added and the benzene-ether layer washed and dried with sodium sulphate.

On removal of the solvents there remained a yellow viscous liquid which partly solidified on trituration with ether. A colourless solid was collected and carefully washed free from oil by means of a little ether.

The solid thus obtained was separated into two constituents, one (A) insoluble and the other (B) soluble, in chloroform. If, as sometimes happened, some unchanged anisil was observed to be present in the mixture (long greenish needles), the anisil and (B) were removed by solution in boiling alcohol, in which (A) is almost insoluble. In such a case (B) was separated from anisil by treatment with a small quantity of pyridine which dissolved the anisil.

The white solid (A) was crystallized from ethyl acetate, being so obtained as shining colourless prisms, m.p. 193–194° (2 g.). It proved to be 3:4-dianisylhexan-3:4-diol, insoluble in cold benzene or ethyl acetate (Found: C, 72.6; H, 7.9.  $C_{20}H_{26}O_4$  requires C, 72.7; H, 7.9 %).

The white solid (B) was recrystallized several times from alcohol and formed colourless prisms, m.p. 105–107° (8 g.). Analysis indicates that the substance is ethylanisylanisoyl carbinol; it is soluble in most organic solvents (Found: C, 72.0; H, 6.7.  $C_{18}H_{20}O_4$  requires C, 72.0; H, 6.7 %).

The oil remaining after removal of the crystalline solids was distilled *in vacuo*. Some anisil sublimed, followed by a light yellow, green-fluorescent, extremely viscous oil, b.p. 215–220°/0.25 mm. (11 g.), evidently 1:2-dianisylbutan-1:2-diol (Found: C, 72.0; H, 7.4.  $C_{18}H_{22}O_4$  requires C, 71.5; H, 7.3 %). It crystallised from alcohol as long prisms, m.p. 96–7°.

Attempts to improve the total yields by replacing most of the ether by benzene (Ramart-Lucas and Legagneur 1929) or heating for 11–25 hr. (Roger 1925) failed, only the quantity of dianisylbutandiol being increased in this way.

The required pinacol was also prepared by heating *p*-methoxypropio-phenone (45 g.) with magnesium amalgam (100 g.) on the steam-bath for 7 days. On acidification and extraction with benzene, an oil was obtained from which the pinacol crystallized (8 g.); the remainder of the ketone was recovered unchanged (35 g.). On recrystallization from ethyl acetate, the pinacol formed colourless prisms of m.p. 192–195° alone or mixed with the pinacol prepared as above, but the fused material was not quite transparent. It may be that a trace of a  $\beta$ -form is present in the product (cf. Ramart-Lucas and Legagneur 1929).

*$\alpha$ -Ethyldeoxyanisoïn.* Deoxyanisoïn (90 g.) and ethyl iodide (54.8 g.) were added to a hot solution of sodium (8.1 g.) in alcohol (150 c.c.). On shaking, a violent reaction set in, necessitating immediate cooling. It is essential that the reaction should proceed rapidly as otherwise the product con-

tained unchanged deoxyanisoin. The solution was refluxed on the steam-bath and in 5–10 min. it showed a neutral reaction. A further quantity of sodium (3 g.) in alcohol (50 c.c.) was added, followed by ethyl iodide (20.4 g.). The whole was heated until neutral (*ca.* 5 hr.). The solution was poured into water and the alcohol removed by distillation under reduced pressure. The cooled solution was extracted with ether, the extracts washed with sodium thiosulphate solution and finally with water. After removal of the solvent, the product distilled as a pale yellow oil, b.p. 189–195°/0.65 mm.; redistilled as a colourless oil, b.p. 192–195°/0.65 mm. (yield, 90 g.) (Found: C, 76.1; H, 6.9.  $C_{18}H_{20}O_3$  requires C, 76.1; H, 7.0 %).

3:4-*Dianisylhexan-3-ol*. A solution of ethyldeoxyanisoin (38.5 g.) was added dropwise to a Grignard solution prepared from ethyl bromide (44.3 g.) and magnesium (9.9 g.) in dry ether (500 c.c.). When the addition was complete, the mixture was heated for 2 hr. on the steam-bath, cooled, and decomposed with ice and dilute sulphuric acid. The ethereal layer was separated, the aqueous layer extracted with further quantities of ether, and the combined ethereal extract washed twice with water and dried with sodium sulphate. The ether was removed and the product, which solidified, crystallized from alcohol in plates, m.p. 115–117°. It could be distilled as a colourless oil, b.p. 194–196°/0.8 mm. (38 g.) (Found: C, 76.5; H, 8.4.  $C_{20}H_{26}O_3$  requires C, 76.4; H, 8.3 %).

Its *p*-nitrobenzoate was obtained by heating it (2.94 g.) with *p*-nitrobenzoyl chloride (3.8 g.) and pyridine (10 c.c.) for 4 hr. on the steam-bath. The mixture was poured into dilute hydrochloric acid, extracted with ether, and the ethereal solution washed with aqueous sodium carbonate. The oil remaining after removal of the solvent was extracted with light petroleum from which shining golden needles separated. The derivative crystallized from alcohol as colourless plates, m.p. 120–122°.

4:4'-*Dimethoxy- $\alpha$ : $\beta$ -diethylstilbene*. Solutions of the above carbinol (36 g.) in chloroform (75 c.c.) and of phosphorus tribromide (31 g.) in chloroform (50 c.c.), were cooled below 0°, mixed, kept overnight at room temperature, then cooled to 0° and poured slowly into ice-cold alcohol (150 c.c.). The alcoholic solution was diluted with water and the chloroform layer separated. The aqueous layer was extracted several times with chloroform and the combined extracts washed with water and dried over calcium chloride. The chloroform was removed and, on cooling, part of the product crystallized; this was collected and washed with light petroleum (12 g.). The mother liquor was heated to about 120° under reduced pressure in order to remove by-products and the residual oil distilled, b.p. 163–166°/0.6 mm. (23 g.). On cooling, it solidified partly to give a further 15 g. of

solid. The colourless oil remaining continued to solidify on keeping, giving more of the same product.

All the solid thus obtained was recrystallized from light petroleum (b.p. 60–80°) and obtained as shining, colourless plates, m.p. 123–124° (total yield, 28–30 g.) (Found: C, 80.8; H, 8.1.  $C_{20}H_{24}O_2$  requires C, 81.0; H, 8.1 %).

The same substance was obtained when the above carbinol (25 g.) was heated with potassium hydrogen sulphate (10 g.) for 10 min. at 195–200°. The water formed was removed by distillation under diminished pressure and the product distilled, b.p. 150–155°/0.2 mm. (22 g.). On cooling, some solid stilbene was obtained (8 g.), the remainder being liquid (13.6 g.).

A boiling mixture of acetyl chloride and acetic anhydride was also employed to dehydrate the carbinol.

The liquid dimethoxydiethylstilbene is doubtless an *isostilbene* (*cis*-form). It was completely freed from the solid by trituration with light petroleum at –10°, filtration from the solid that separated, evaporation of the solution and several repetitions of the process until no more solid could be obtained. The oil was washed in ethereal solution with aqueous sodium carbonate and water, the solution dried and distilled, b.p. 175–178°/0.74 mm.,  $n_D^{18}$ , 1.5699. The oil was stable in the dark but when exposed to sunlight it was gradually transformed into the solid modification already described.

4:4'-*Dihydroxy- $\alpha$ : $\beta$ -diethylstilbene* (III). Attempts were also made to bring about demethylation of the dimethoxydiethylstilbene by means of aluminium chloride and aluminium bromide. Light petroleum (20 c.c. of b.p. 60–80°) was used as solvent for the stilbene (3 g.), which was heated with aluminium chloride (6 g.) for 6 hr. at 60°. The light petroleum was decanted and the residue decomposed with ice, acidified with hydrochloric acid, and extracted with ether. The ethereal extract was shaken with sodium hydroxide solution and this was acidified and extracted with ether. A liquid was finally obtained from which phenol and other phenolic products were obtained, together with a very small amount of a brown oil of b.p. 145–153°/0.8 mm. The use of aluminium bromide was no more successful.

The above crystalline dimethyl ether (20 g.) was heated with potassium hydroxide (50 g.) and alcohol (200 c.c.) in a constantly rotating autoclave for 24 hr. at 200–210° (the maximum pressure was about 15 atm.). The contents of the autoclave were diluted with water and the alcohol evaporated. After cooling, the solution was extracted with ether, filtered, heated to remove ether, cooled, and acidified with dilute hydrochloric acid. The colourless crystalline solid was filtered and recrystallized from benzene-light petroleum (yield, 16 g.); no unchanged material was recovered. The substance crystal-

lized well from 50 % alcohol, benzene, benzene-light petroleum, or ethylene chloride. It usually had m.p. 167–168° but a specimen recovered from the pure dipropionate had m.p. 171° (Found: C, 80.8; H, 7.7; MeO, 0.0.  $C_{18}H_{20}O_2$  requires C, 80.6; H, 7.7 %).

The *diacetate* was prepared by boiling the dihydroxydiethylstilbene (0.75 g.) with acetic anhydride (1.5 c.c.) and pyridine (3 c.c.) for 5 min., pouring into water, and crystallizing the solid which separated from alcohol. It formed thick needle-like prisms, m.p. 123–124° (0.6 g.) (Found: C, 75.0; H, 6.8.  $C_{22}H_{24}O_4$  requires C, 75.0; H, 6.8 %).

The *dipropionate*, obtained by the action of an excess of propionic anhydride and a few drops of pyridine on diethylstilboestrol at 100°, crystallized from methyl alcohol in colourless plates, m.p. 104° (Found: C, 75.7; H, 7.4.  $C_{24}H_{28}O_4$  requires C, 75.8; H, 7.4 %).

The *di-n-butyrate*, prepared in a similar manner, crystallized from methyl alcohol in flat needles, m.p. 88° (Found: C, 76.3; H, 7.9.  $C_{26}H_{32}O_4$  requires C, 76.5; H, 7.8 %).

The *di-isobutyrate* formed colourless, elongated plates, m.p. 86–87° (Found: C, 76.5; H, 7.8 %).

The *di-n-valerate*, also obtained by the use of the acid anhydride and pyridine, crystallized from light petroleum (b.p. 40–60°) in flat needles, m.p. 89° (Found: C, 77.0; H, 8.3.  $C_{28}H_{36}O_4$  requires C, 77.1; H, 8.2 %).

The *di-palmitate*. Diethylstilboestrol (1.0 g.) in pyridine (6 c.c.) was shaken with palmityl chloride (3.0 g.) and allowed to stand overnight at room temperature. The mixture was then shaken with ether and dilute hydrochloric acid. The ethereal solution was washed with sodium hydroxide solution and water, dried and evaporated. The ester was crystallized from boiling alcohol, in which it is only sparingly soluble; colourless plates, m.p. 77–78° (Found: C, 79.9; H, 10.7.  $C_{50}H_{80}O_4$  requires C, 80.6; H, 10.8 %).

The *dibenzoate* crystallized from ethyl acetate or ethyl acetate-alcohol (1 : 1) in needles, m.p. 210–211° (Found: C, 80.4; H, 5.8.  $C_{32}H_{28}O_4$  requires C, 80.7; H, 5.9 %).

The *di- $\alpha$ -naphthoate*, prepared by action of  $\alpha$ -naphthoyl chloride in pyridine solution, crystallized from ethyl acetate in prisms, m.p. 206–207° (Found: C, 83.0; H, 5.8.  $C_{40}H_{32}O_4$  requires C, 83.3; H, 5.6 %).

The *di- $\beta$ -naphthoate* crystallized from ethyl acetate in prismatic needles, m.p. 252–253° (Found: C, 83.1; H, 5.6 %).

The *di-phenylacetate*, prepared by the action of phenylacetyl chloride on a solution of diethylstilboestrol in dry pyridine at 0°, crystallized from alcohol in colourless needles, m.p. 100° (Found: C, 80.7; H, 6.4.  $C_{34}H_{32}O_4$  requires C, 81.0; H, 6.3 %).



*$\psi$ -Diethylstilboestrol.* The liquid modification of the dimethyl ether (20 g.) was heated in a rotating autoclave with potassium hydroxide (50 g.) and alcohol (200 c.c.) for 24 hr. The product worked up as before gave diethylstilboestrol, m.p. 167–168° (7.5 g.) after five crystallizations from benzene. The mother liquors were diluted with light petroleum and the solid so precipitated was crystallized thrice from ethylene chloride and thrice from benzene; colourless prismatic needles, m.p. 140–142° and m.p. 130–147° when mixed with diethylstilboestrol (Found: C, 80.7; H, 7.7; MeO, 0.0.  $C_{18}H_{20}O_2$  requires C, 80.6; H, 7.7 %).

The *di-benzoate*, crystallized from ethyl acetate, had m.p. 193–197° and the *di-acetate*, crystallized from alcohol had m.p. 116–117° (Found: C, 75.2; H, 6.9.  $C_{22}H_{24}O_4$  requires C, 75.0; H, 6.8 %).

4:4'-*Dihydroxy- $\alpha$ : $\beta$ -diethyldiphenylethane*.\* (A) A solution of the dimethoxydiethylstilbene (4 g.) in glacial acetic acid (10 c.c.) and hydriodic acid (40 c.c., d. 1.94) was heated at 135–140° for 1 hr. The mixture was poured into water, clarified with sulphur dioxide, and extracted with ether. On distillation, a viscous oil resulted, having b.p. 189–190°/0.8 mm.; it hardened on cooling (Found: C, 79.9; H, 8.4.  $C_{18}H_{22}O_2$  requires C, 80.0; H, 8.2 %). This material is undoubtedly a mixture.

(B) A *product* of unknown constitution was obtained when the pinacol from *p*-methoxypropiofenone was boiled with concentrated hydriodic acid in the presence of red phosphorus. The substance crystallized from light petroleum (b.p. 60–80°) as colourless, prismatic needles, m.p. 64.5–65° (Found: C, 74.9; H, 8.3.  $C_{18}H_{22}O_2$ ,  $H_2O$  requires C, 75.0; H, 8.4 %). This material was oestrogenic in doses on 0.01 g.

(C) A solution of  *$\psi$ -diethylstilboestrol* (3 g.) in alcohol (25 c.c.) was shaken with a platinum oxide catalyst (0.1 g.) in presence of hydrogen at the room temperature for 36 hr. Absorption of hydrogen was rapid at first but soon slowed down considerably. When about 1300 c.c. had been taken up, the reduction ceased (required for 7 double bonds, 1900 c.c.). On removal

[Footnote added in proof. In the interval between submission of this communication and correction of the proofs the facts in connection with the reduction of diethylstilboestrol have been further elucidated. Using palladized charcoal as the catalyst the sole product is found to be dihydroxydiphenylhexane, m.p. 128°. Pseudodiethylstilboestrol gives the isomeride, m.p. 185°, along with some diethylstilboestrol (isomeric change) and hence also a small proportion of the isomeride, m.p. 128°. The dimethyl ethers of both diethylstilboestrol and pseudodiethylstilboestrol give one and the same product in excellent yield. This is the dimethoxydiphenylhexane, m.p. 145–146°, which on dimethylation by means of hot hydriodic acid or hydrobromic acid affords the dihydroxydiphenylhexane, m.p. 185°, in good yield. Methylation of the dihydroxydiphenylhexane, m.p. 128°, gives the dimethyl ether, m.p. 56–57° (Found: C, 80.4; H, 8.6  $C_{18}H_{22}O_2$  requires C, 80.5; H, 8.7%).]

of the alcohol and distillation of the residual oil there resulted: (a) a colourless viscous oil, b.p. 170–175°/13 mm. (1.8 g.); (b) an oil which solidified on cooling, b.p. 210–230°/13 mm.; (c) a residual solid which crystallized from benzene and proved to be unchanged diethylstilboestrol.

(a) The oil was redistilled, b.p. 184–187°/21 mm. It was saturated, and was insoluble in alkaline solutions (Found: C, 81.6; H, 11.0 %).

(b) The solid was recrystallized from benzene and had m.p. 178–180°. Recrystallized twice more, it had m.p. 181–182°. It was saturated (Found: C, 80.1; H, 8.2.  $C_{18}H_{22}O_2$  requires C, 80.0; H, 8.2 %).

(D) Diethylstilboestrol (2 g.) was hydrogenated in acetone solution in the presence of a palladium catalyst. Rather less than the calculated volume of hydrogen was absorbed in 60 hr. Repeated crystallization from benzene gave 0.3 g., mp. 184°. From the mother liquor, after several crystallizations from light petroleum or benzene-light petroleum, a small amount of a substance melting at 128° was obtained (Found: C, 80.0; H, 8.2.  $C_{18}H_{22}O_2$  requires C, 80.0; H, 8.2 %).

In a subsequent similar experiment the product consisted almost entirely of the lower melting isomeride, the oestrogenic activity of which was much less than that of the higher melting form. It produced full response in all rats in doses of 100γ; doses of 10γ showed little activity.

(E) 4:4'-Dihydroxy-γ:δ-diphenylhexadiene (0.5 g.) was hydrogenated in acetone solution in the presence of a palladium catalyst. The required volume of hydrogen was taken up in 45 min. The product crystallized from benzene in colourless needles, m.p. 185°, and the yield was almost quantitative. This specimen was identical with the active product of demethylation of anethole which was analysed in the course of the investigation of Campbell, Dodds and Lawson (1938), a fuller account of which will be published later.

4:4'-Dihydroxy-α-ethyldeoxybenzoin. Hydriodic acid (100 c.c. of d. 1.7) was added to a solution of ethyldeoxyanisoin (20 g.) in glacial acetic acid (20 c.c.). The mixture was gradually heated to 135–140°, maintained at that temperature for 15 min., and then poured into water. The liquid was clarified with sulphur dioxide and extracted with ether. On removal of the ether, a pale yellow, slightly opalescent oil remained (17 g.); on distillation it had b.p. 210–215°/0.6 mm. and set to a glass on cooling (15 g.) (Found: C, 74.9; H, 6.3.  $C_{16}H_{16}O_3$  requires C, 75.0; H, 6.2 %).

The acetate was prepared as described above for diethylstilboestrol; the derivative crystallized from alcohol in needles, m.p. 91–92°.

Dibenzoate of 4:4'-dihydroxy-α-ethyldeoxybenzoin. The dihydroxy compound (15 g.) was dissolved in sodium hydroxide solution (100 c.c. of 20 %)

and benzoyl chloride (18 g.) gradually added with cooling and vigorous shaking. On keeping, a solid separated which was collected, dried, and recrystallized from ethyl acetate or ethyl acetate-alcohol. It formed small needles of m.p. 138–140° (19.6 g.) (Found: C, 77.5; H, 5.2.  $C_{30}H_{24}O_6$  requires C, 77.6; H, 5.2 %).

*Conversion to diethylstilboestrol.* A Grignard solution was prepared from ethyl bromide (21.1 g., 5 mol.) and magnesium (4.7 g., 5 mol.) in dry ether (300 c.c.). The above dibenzoate (18 g.) was added slowly, with vigorous stirring. When the addition was complete, the mixture was heated for 2 hr. on the steam-bath, cooled, and decomposed with ice and dilute sulphuric acid. The ethereal layer yielded an oil which was heated to a temperature of 150° at approximately 0.3 mm. pressure, when a colourless liquid distilled. The residual brown glass was dissolved in benzene (50 c.c.) and on cooling, colourless prisms separated, which were recrystallized from benzene (yield, 4 g. of m.p. 168°). From the mother liquors a further 4 g. of less pure product was obtained. The product was in every way identical with that obtained by the method described above. The dibenzoates of the two products were also identical.

*Dibenzyl ether of 4:4'-dihydroxyethyldeoxybenzoin.* The method of Allan and Robinson (1926) was employed. A solution of the keto-phenol (2 g.) in alcohol (50 c.c.) was heated with benzyl chloride (2.25 g.) and potassium hydroxide (1.1 g.) for 1 hr. Dilution with water caused the separation of an oil which solidified on standing. It crystallized from light petroleum in leaflets, m.p. 78–80° (1.6 g.) (Found: C, 82.5; H, 6.3.  $C_{30}H_{28}O_3$  requires C, 82.6; H, 6.4 %).

*3:4-Di-(p-benzylloxyphenyl)-hexan-3-ol.* The benzyl ether (1.5 g.) was gradually added to a warm Grignard solution prepared from magnesium (0.4 g.) and ethyl bromide (1.9 g.) in dry ether (50 c.c.). A vigorous reaction, accompanied by the momentary production of a red colour, resulted. After heating on the steam-bath for 2 hr., the mixture was decomposed with ice and dilute sulphuric acid. A white crystalline solid that separated was collected and combined with more of the solid obtained from the ethereal layer. By extraction and crystallization from boiling methyl alcohol, colourless prisms were obtained, m.p. 142–144° (1.2 g.) (Found: C, 82.3; H, 7.3.  $C_{32}H_{34}O_3$  requires C, 82.4; H, 7.3 %). A small portion of the product was found to be insoluble in boiling methyl alcohol; it was crystallized from chloroform and obtained as small prisms, m.p. 212–214° (Found: C, 82.1; H, 7.3.  $C_{32}H_{34}O_3$  requires C, 82.4; H, 7.3 %). The substance is evidently a *stereoisomeride* of the more fusible compound first described.

*Conversion to the diacetate of diethylstilboestrol.* The carbinol was treated

with phosphorus tribromide in chloroform in the manner described above. A yellow oil resulted which on distillation yielded benzyl bromide and a glassy residue. This was soluble in alkali, but could not be induced to crystallize. On acetylation with acetic anhydride and pyridine it gave an oil which was taken up in ether, and washed with aqueous sodium carbonate. On removal of the ether, the substance solidified on trituration with alcohol. It crystallized from alcohol in prisms of m.p.  $123-124^{\circ}$ ; the m.p. showed no depression on admixture of the product with an authentic specimen of the diacetate of diethylstilboestrol.

*1:2-Dianisylbutan-2-ol.* Deoxyanisoin (7.7 g.) was added slowly to a Grignard solution from magnesium (2.2 g.) and ethyl bromide (9.8 g.) in dry ether (150 c.c.). The mixture was heated for 2 hr. on the steam-bath, cooled, and decomposed with ice and dilute sulphuric acid. On working up the product, a liquid of b.p.  $178-181^{\circ}/0.6$  mm. resulted. It solidified on cooling and was recrystallized from light petroleum, m.p.  $61-62^{\circ}$  (Found: C, 75.5; H, 7.8.  $C_{18}H_{22}O_3$  requires C, 75.5; H, 7.7 %). (Yield, 7.8 g.)

*4:4'-Dimethoxy- $\alpha$ -ethylstilbene.* The carbinol (14.3 g.) was dehydrated with phosphorus tribromide in the manner described above. The colourless oil of b.p.  $165-166^{\circ}/0.75$  mm. that resulted solidified almost completely and crystallized from light petroleum and from alcohol in shining prisms, m.p.  $85^{\circ}$  (Found: C, 80.5; H, 7.5.  $C_{18}H_{20}O_2$  requires C, 80.6; H, 7.5 %). (Yield, 11.5 g.)

The carbinol (10 g.) was also dehydrated with potassium hydrogen sulphate, the yield of the stilbene derivative being 9.1 g. A mixture of acetyl chloride and acetic anhydride could also be employed for the same purpose.

*4:4'-Dihydroxy- $\alpha$ -ethylstilbene.* The dimethoxyethylstilbene (10 g.) was demethylated with potassium hydroxide and alcohol as described above. Distillation of the isolated product gave a pale yellow glassy oil, b.p.  $208-211^{\circ}/0.3$  mm. (7.5 g.). This solidified and crystallized from benzene-light petroleum in small plates, m.p.  $128-129^{\circ}$  (Found: C, 80.5, H, 6.8.  $C_{18}H_{18}O_2$  requires C, 80.0; H, 6.7 %).

The *dibenzoate*, short needles from alcohol or light petroleum, had m.p.  $100-102^{\circ}$  (Found: C, 80.3; H, 5.5.  $C_{30}H_{24}O_4$  requires C, 80.4; H, 5.4 %).

*$\alpha$ -Methyldeoxyanisoin.* Deoxyanisoin (51.2 g.) and methyl iodide (28.4 g.) were added to a solution of sodium (4.6 g.) in alcohol (100 c.c.), and the mixture cooled in ice. The reaction became neutral almost immediately and a further quantity of sodium (4.6 g.) in alcohol (75 c.c.) and of methyl iodide (28.4 g.) were added, heating on the steam-bath for 20 min. being sufficient to complete the reaction. Water was added and the product

isolated in the usual manner. The oil obtained had b.p.  $176-177^{\circ}/0.1$  mm. (51.5 g.); it crystallized from light petroleum as prisms, m.p.  $53-57^{\circ}$  (Found: C, 75.5; H, 6.5.  $C_{17}H_{15}O_3$  requires C, 75.5; H, 6.7 %).

**2:2-Dianisylbutan-2-ol.** A solution of  $\alpha$ -methyldeoxyanisoin (27 g.) in ether (150 c.c.) was added to a solution of magnesium (7.3 g.) and methyl iodide (42.6 g.) in ether (200 c.c.). After heating for 2 hr. on the steam-bath, the product was isolated in the usual manner. It crystallized from light petroleum or alcohol, m.p.  $87-89^{\circ}$  (28 g.) (Found: C, 75.5; H, 7.8.  $C_{18}H_{22}O_3$  requires C, 75.5; H, 7.7 %).

**4:4'-Dimethoxy- $\alpha$ : $\beta$ -dimethylstilbene.** The carbinol (27.5 g.) was dehydrated to the stilbene with phosphorus tribromide (26 g.). The product crystallized from alcohol in prisms, m.p.  $127-129^{\circ}$  (23 g.) (Found: C, 80.3; H, 7.4.  $C_{18}H_{20}O_2$  requires C, 80.6; H, 7.4 %).

**4:4'-Dihydroxy- $\alpha$ : $\beta$ -dimethylstilbene (dimethylstilboestrol).** The above stilbene (3 g.) was demethylated in a sealed tube at  $200-210^{\circ}$  with potassium hydroxide (7.5 g.) in alcohol (20 c.c.). The product crystallized from benzene-light petroleum or ethylene chloride in prisms, m.p.  $194-196^{\circ}$  (Found: C, 80.0; H, 6.8.  $C_{16}H_{16}O_2$  requires C, 80.0; H, 6.7 %). The mother liquor of the crystallization on dilution with light petroleum gave the *monomethyl ether* of dimethylstilboestrol; wide plates, m.p.  $115-116^{\circ}$  (Found: C, 80.1; H, 7.0.  $C_{17}H_{18}O_2$  requires C, 80.3; H, 7.0 %). The methoxyl content was somewhat higher than the theoretical.

**4:4'-Dimethoxy- $\alpha$ -methyl- $\beta$ -ethylstilbene.** Methyldeoxyanisoin (20 g.) in ether (100 c.c.) was added to a solution of magnesium (5.4 g.) and ethyl iodide (34.6 g.) in ether (150 c.c.) and the mixture heated for 2 hr. The product was a colourless oil, b.p.  $159-161^{\circ}/0.14$  mm. (Found: C, 80.8; H, 7.8.  $C_{19}H_{22}O_2$  requires C, 80.9; H, 7.8 %). (Yield, 19 g.)

**Methylethylstilboestrol.** The above dimethoxymethylethylstilbene (8 g.) was demethylated in a rotating autoclave at  $200-210^{\circ}$  with potassium hydroxide (20 g.) and alcohol (150 c.c.). The product was a brown oil which gave a sparingly soluble sodium salt. From this, on acidification of a dilute aqueous solution, a colourless solid was obtained. Three crystallizations from benzene gave colourless clusters of needles, m.p.  $179-180^{\circ}$ , turning a light red in sunlight (Found: C, 80.1; H, 7.3.  $C_{17}H_{18}O_2$  requires C, 80.3; H, 7.1 %). The alkaline solution from which the sodium salt had been separated was shaken with benzoyl chloride. The brown oil which separated solidified on trituration with alcohol and was crystallized from ethyl acetate. It formed prisms, m.p.  $217-219^{\circ}$  (Found: C, 80.4; H, 5.7.  $C_{31}H_{28}O_4$  requires C, 80.5; H, 5.6 %).

**$\alpha$ -n-Propyldeoxyanisoin.** Deoxyanisoin (51 g.) did not react very rapidly

with *n*-propyl iodide (34 g.) and sodium (4.6 g.) in alcohol (100 c.c.). The solution became neutral in reaction after  $\frac{3}{4}$  hr., when sodium (2.3 g.) in alcohol (50 c.c.) and *n*-propyl iodide (17 g.) were added and the heating continued for a further period of 3 hr. On working up, unchanged deoxyanisoin was recovered (12.5 g.) and a colourless oil obtained, having b.p. 195–196°/0.14 mm. (35 g.) (Found: C, 76.7; H, 7.5 %.  $C_{19}H_{22}O_3$  requires C, 76.5; H, 7.4 %).

3:4-*Dianisylheptan-3-ol*. *n*-Propyldeoxyanisoin (8 g.) in ether (75 c.c.) was added to a Grignard solution from magnesium (2 g.) and ethyl bromide (8.8 g.). The product, isolated in the usual way, was a greenish yellow jelly of b.p. 176–177°/0.3 mm. (Found: C, 77.0; H, 8.3.  $C_{21}H_{28}O_3$  requires C, 76.8; H, 8.5 %). (Yield, 8.8 g.)

4:4'-*Dimethoxy- $\alpha$ -ethyl- $\beta$ -*n*-propylstilbene* resulted on dehydration of the carbinol (8 g.) with phosphorus tribromide. It was also obtained directly by the action of *n*-propyl magnesium bromide in excess on ethyldeoxyanisoin. It had b.p. 192–195°/0.4 mm. (Found: C, 81.1; H, 8.3.  $C_{21}H_{26}O_2$  requires C, 81.2; H, 8.4 %).

*Ethyl n-propylstilboestrol*. On demethylation of the above stilbene derivative (11 g.) in a rotating autoclave under the usual conditions, a pale yellow oil, b.p. 198–200°/0.14 mm. resulted (Found: C, 80.7; H, 7.8.  $C_{19}H_{22}O_2$  requires C, 80.8; H, 7.8 %). (Yield, 8.5 g.)

The dibenzoate crystallized from alcohol, or benzene-light petroleum in prismatic needles, m.p. 208–211°.

4:4'-*Dimethoxy- $\alpha$ : $\beta$ -di-*n*-propylstilbene*. *n*-Propyldeoxyanisoin (20 g.) on treatment with *n*-propyl magnesium bromide gave a mixture of carbinol and stilbene (18 g.). After treatment with phosphorus tribromide the b.p. was 178–181°/0.8 mm. (Found: C, 81.1; H, 8.3.  $C_{22}H_{28}O_2$  requires C, 81.5; H, 8.6 %). Some 4:4'-dimethoxystilbene, m.p. 214°, was also obtained.

*Di-*n*-propylstilboestrol*. The dimethyl ether (8 g.) was demethylated in the usual way, giving 5 g. of a pale yellow oil, b.p. 198–201°/0.09 mm. (Found: C, 81.2; H, 8.2.  $C_{20}H_{24}O_2$  requires C, 81.0; H, 8.1 %).

*$\alpha$ -iso-Propyldeoxyanisoin*. Deoxyanisoin (38.4 g.), isopropyl iodide (25.5 g.) and sodium (3.45 g.) in alcohol (75 c.c.) were heated for 12 hr. After addition of the same quantity of sodium in alcohol and isopropyl iodide as before, the heating was continued for 12 hr. The product, which contained unchanged deoxyanisoin (10 g.), was otherwise a pale yellow oil, b.p. 210–214°/0.8 mm. (24 g.) (Found: C, 76.5; H, 7.5.  $C_{19}H_{22}O_3$  requires C, 76.5; H, 7.4 %).

2:5-*Dimethyl-3:4 dianisyl-hexan-3-ol*. *iso*-Propyldeoxyanisoin (29.8 g.) on treatment with isopropyl magnesium bromide gave a yellow jelly-like

oil, b.p. 205–207°/0.27 mm. (23.5 g.) (Found: C, 77.1; H, 8.9.  $C_{22}H_{30}O_3$  requires, C, 77.2; H, 8.8 %).

*4:4'-Dimethoxy- $\alpha$ : $\beta$ -di-iso-propylstilbene.* The carbinol (23.5 g.) was dehydrated with potassium hydrogen sulphate, giving a pale yellow oil, b.p. 181–182°/0.25 mm. (20 g.). From the oil there crystallized a small quantity of 4:4'-dimethoxystilbene, m.p. 214°; it was filtered and redistilled (Found: C, 81.3; H, 8.5.  $C_{22}H_{28}O_2$  requires C, 81.5; H, 8.6 %).

*Di-iso-propylstilboestrol.* Demethylation of the above stilbene derivative (15 g.) gave a yellow oil of b.p. 202–204°/0.25 mm. (6 g.) (Found: C, 80.8; H, 8.0.  $C_{20}H_{24}O_2$  requires C, 81.0; H, 8.1 %).

The *dibenzoate* crystallized from ethyl acetate in shining rectangular prisms, m.p. 155° (Found: C, 80.9; H, 6.3.  $C_{34}H_{32}O_4$  requires C, 81.0; H, 6.3 %).

*$\alpha$ -n-Butyldeoxyanisoin.* Deoxyanisoin (35 g.) was *n*-butylated during 24 hr. in the usual manner (recovered deoxyanisoin, 8.5 g.). The product was a pale yellow liquid of b.p. 205–206°/0.6 mm. (26.2 g.) (Found: C, 76.6; H, 7.5.  $C_{20}H_{24}O_3$  requires C, 76.9; H, 7.7 %).

*4:4'-Dimethoxy- $\alpha$ : $\beta$ -di-*n*-butylstilbene.* *n*-Butyldeoxyanisoin (15.6 g.) on treatment with *n*-butyl magnesium bromide gave a colourless oil, b.p. 206–208°/0.6 mm. Some 4:4'-dimethoxystilbene crystallized and was collected. The oil was redistilled, b.p. 186–188°/0.16 mm. (Found: C, 81.5; H, 9.0.  $C_{24}H_{32}O_2$  requires C, 81.8; H, 9.2 %).

*Di-*n*-butylstilboestrol.* Demethylation of the above stilbene derivative gave an oil, b.p. 191–196°/0.2 mm. (Found: C, 81.2; H, 8.8.  $C_{22}H_{28}O_2$  requires C, 81.5; H, 8.6 %).

The *dibenzoate* crystallizes from ethyl acetate in hexagonal plates, m.p. 192–193° (Found: C, 80.8; H, 6.8.  $C_{36}H_{36}O_4$  requires C, 81.2; H, 6.8 %).

*4:4'-Dimethoxy- $\alpha$ -ethyl- $\beta$ -allylstilbene.* Magnesium (7.3 g.), ethyldeoxyanisoin (28.4 g.) and ether (150 c.c.) were heated and stirred, reaction being initiated with a little methyl iodide. A solution of allyl bromide (60.5 g.) in ether (200 c.c.) was added dropwise over a period of 2 hr. and the mixture heated for 3 hr. A yellow oil, obtained on working up the product, had b.p. 197–198°/0.8 mm. (30.5 g.) (Found: C, 81.6; H, 7.8.  $C_{21}H_{24}O_2$  requires C, 81.7; H, 7.8 %).

*Ethylpropenylstilboestrol.* The above stilbene derivative was demethylated with alcoholic potassium hydroxide in a rotating autoclave. The product was a pale yellow oil, b.p. 208–211°/0.17 mm. (Yield from 12 g., 6.7 g.) (Found: C, 81.2; H, 6.9.  $C_{19}H_{20}O_2$  requires C, 81.4; H, 7.1 %). We have made the probable assumption that the double bond of the allyl group migrates into the conjugated position under the influence of hot alcoholic potassium

hydroxide. The same view has been adopted in other cases. An analogy is the conversion of safrole (methylenedioxyallylbenzene) into *isosafrole* (methylenedioxyisopropenylbenzene) under the influence of alkaline catalysts.

The *dibenzoate* crystallized from alcohol in needles, m.p. 111–113° (Found: C, 80.9; H, 5.8.  $C_{33}H_{28}O_4$  requires C, 81.1; H, 5.8 %).

*α-Allyldeoxyanisoin*. Deoxyanisoin (51.2 g.) was added to a hot solution of sodium (4.6 g.) in alcohol (80 c.c.). Allyl bromide (48.4 g.) was added slowly, with cooling to control the ensuing very vigorous reaction. After heating for 2 hr., the alcohol was evaporated under reduced pressure and water and ether added. Deoxyanisoin separated and was collected. The product, isolated from the ethereal layer in the usual manner, was distilled. Trisubstitution of the distillate with ether caused the separation of more deoxyanisoin; this was removed by filtration and the product distilled again as a pale yellow oil, b.p. 196–198°/0.13 mm. (27.3 g.). In order to obtain a pure product for analysis, the process of purification described above was repeated twice more (Found: C, 76.8; H, 6.8.  $C_{19}H_{20}O_3$  requires C, 77.0; H, 6.7). (18.5 g. of deoxyanisoin was recovered.)

*Δ<sup>1:7-4:5</sup>-Dianisyl-octadiene-5-ol*. Allyldeoxyanisoin (29.6 g.) and magnesium (7.3 g.) in gently boiling dry ether (180 c.c.) were stirred and reaction initiated with iodine and a little methyl iodide. Allyl bromide (60.5 g.) was added dropwise over a period of 1 hr., heating being continued for a further period of 2 hr. After decomposition with ice and ammonium chloride, the product was isolated as an extremely viscous, pale yellow oil (23.4 g.), b.p. 198–203°/0.23 mm. (Found: C, 78.2; H, 7.6.  $C_{22}H_{26}O_3$  requires C, 78.2; H, 7.7 %).

*4:4'-Dimethoxy-α:β-diallylstilbene*. The above carbinol (20 g.) and potassium hydrogen sulphate (10 g.) were heated together and the product distilled; it was found to be not entirely homogeneous. It was again heated with a further small quantity of potassium bisulphate and on distillation a pale yellow viscous oil resulted, b.p. 186–188°/0.09 mm. (15.3 g.) (Found: C, 82.3; H, 7.3.  $C_{22}H_{24}O_2$  requires C, 82.5; H, 7.5 %).

*4:4'-Dihydroxy-α:β-dipropenylstilbene*. Demethylation of the above stilbene (14 g.) with alcoholic potassium hydroxide yielded a glass having b.p. 220–226°/0.4 mm. (7 g.) (Found: C, 81.8; H, 7.0.  $C_{20}H_{20}O_2$  requires C, 82.2; H, 6.8 %).

Its *dibenzoate* formed long slender needles from alcohol-ethyl acetate; it had m.p. 164° (Found: C, 81.4; H, 5.4.  $C_{34}H_{28}O_4$  requires C, 81.6; H, 5.6 %).

*2:7-Dimethyl-4:5-dianisyl-octane-2:7-diol-a*. Methyl dianisyladipate-*a* (7.7 g.; Ramage and Robinson 1933) was added in small quantities to a



Grignard solution of methyl iodide (28.4 g.) and magnesium (4.9 g.) in ether (100 c.c.). After heating for 2 hr. on the steam-bath, the solution was decomposed with ice and ammonium chloride. The product isolated was a crystalline solid. It crystallized from chloroform-light petroleum in short slender needles, m.p. 125–126° (8.1 g.) (Found: C, 74.3; H, 8.8.  $C_{24}H_{34}O_4$  requires C, 74.6; H, 8.8 %).

*$\Delta^{2:6-2:7}$ -Dimethyl-4:5-dianisyl-octadiene.* The carbinol (5 g.) was dehydrated by means of potassium hydrogen sulphate (3 g.). The pale rose-coloured oily product exhibited a bluish fluorescence, and had b.p. 202–203°/0.14 mm. (3.6 g.) (Found: C, 82.1; H, 8.5.  $C_{24}H_{30}O_2$  requires C, 82.3; H, 8.6 %).

*2:7-Dimethyl-4:5-dianisyl-octane.* The dimethyldianisyl-octadiene (0.7 g.) in alcohol (10 c.c.) was shaken with hydrogen in presence of Adams' platinum oxide catalyst (0.1 g.); 75 c.c. of hydrogen were absorbed in the course of  $\frac{1}{2}$  hr. On distillation of the product, a reddish yellow glass was obtained; the b.p. was 210–220°/0.3 mm. (Found: C, 82.0; H, 9.0.  $C_{24}H_{32}O_2$  requires C, 81.8; H, 9.1 %).

*$\Delta^{2:6-2:7}$ -Dimethyl-4:5-di-(*p*-hydroxyphenyl)-octadiene.* Demethylation of the above dimethyldianisyl-octadiene (3 g.) with potassium hydroxide (7 g.) in alcohol (25 c.c.) at 200–210° resulted in a thick yellow oil of b.p. 215–220°/0.01 mm. (Found: C, 81.9; H, 8.1.  $C_{22}H_{26}O_2$  requires C, 82.0; H, 8.1 %).

The *dibenzoate* formed colourless prisms from alcohol, m.p. 71–72° (Found: C, 81.6; H, 6.2.  $C_{36}H_{34}O_4$  requires C, 81.5; H, 6.4 %).

*4:4'-Dimethoxy- $\alpha$ -phenyl stilbene.* Deoxyanisoin (5 g.) was added gradually to an ice-cold Grignard solution prepared from bromobenzene (9.3 g.) and magnesium (1.5 g.) in dry ether (40 c.c.). The mixture was heated for 2 hr. on the steam-bath and decomposed with ice and ammonium chloride. The ether layer was separated, washed with water, dried and evaporated. The residue solidified and crystallized from alcohol in small prisms, m.p. 111–112° (5.0 g.). This substance is doubtless  $\alpha\beta$ -dianisyl- $\alpha$ -phenylethanol.

The carbinol (9 g.) was boiled for 8 hr. with a mixture of acetyl chloride (10 c.c.) and acetic anhydride (20 c.c.); the mixture was then decomposed with water and extracted with ether. The ethereal solution was washed with sodium carbonate solution and water, dried and evaporated and the residue was crystallized from alcohol. These crystals (6.3 g.) were dissolved in hot alcohol (100 c.c.) and on slow cooling the solution deposited large prismatic crystals, m.p. 101–103° (3.3 g.), raised by recrystallization from alcohol and finally from light petroleum to 105–106° (Found: C, 83.7; H, 6.3.  $C_{22}H_{30}O_2$  requires C, 83.5; H, 6.4 %). On shaking, the mother liquor gave a further crop of smaller crystals (2.3 g.), m.p. 75–85°, which after

several crystallizations from alcohol and finally from light petroleum had m.p. 92–93° (Found: C, 83.6; H, 6.3%). A mixture of the two forms melted at 75–80°.

*4:4'-Dihydroxy- $\alpha$ -phenyl-stilbene.* This substance was prepared by demethylation of the above dimethyl ether by means of potassium hydroxide and alcohol in a sealed tube at 200° for 15 hr. Both forms of the dimethyl ether gave the same product, which after crystallization from chloroform and finally from benzene had m.p. 99–100° (Found: C, 83.8; H, 5.5.  $C_{20}H_{16}O_2$  requires C, 83.3; H, 5.6 %).

*4:4'- $\alpha\beta$ -Tetrahydroxy- $\alpha\beta$ -diethyl- $\alpha\beta$ -diphenylethane.* *p*-Hydroxypropio-phenone (20 g.) was reduced in moist ether by the amalgam from 40 g. aluminium foil (Vogel 1927). The ethereal solution was filtered from the aluminium hydroxide, dried and evaporated. The residue, dissolved in glacial acetic acid (40 c.c.), slowly deposited crystals of the pinacol (7 g.) which after recrystallization from alcohol melted at 204–206° (Found: C, 71.4; H, 7.35.  $C_{18}H_{22}O_4$  requires C, 71.5; H, 7.3 %).

The *diacetate*, prepared by boiling with acetic anhydride, crystallizes from alcohol and has m.p. 199–200° (Found: C, 68.0; H, 6.9.  $C_{22}H_{26}O_6$  requires C, 68.4; H, 6.9 %).

*4:4'-Diacetoxy- $\gamma$ : $\delta$ -diphenyl- $\beta$ : $\delta$ -hexadiene* was prepared by boiling the above pinacol with a mixture of acetyl chloride and acetic anhydride for 8 hr. The reaction mixture was decomposed with water and extracted with ether. The ethereal solution after washing with sodium carbonate solution and water was dried and evaporated. The residue crystallized from alcohol in small lozenge-shaped prisms, m.p. 119–120° (Found: C, 75.5; H, 6.5.  $C_{22}H_{22}O_4$  requires C, 75.4; H, 6.3 %).

*4:4'-Dihydroxy- $\gamma$ : $\delta$ -diphenyl- $\beta$ : $\delta$ -hexadiene*, obtained by hydrolysis of the acetate, crystallizes from dilute alcohol in fine needles, m.p. 227–228° (Found: C, 81.1; H, 6.8.  $C_{18}H_{18}O_2$  requires C, 81.2; H, 6.8 %).

The *dipropionate* prepared by boiling with propionic anhydride crystallizes from alcohol in prisms, m.p. 96°.

*4:4'- $\alpha\beta$ -Tetrahydroxy- $\alpha\beta$ -di-*n*-propyl- $\alpha\beta$ -diphenylethane.* This pinacol was prepared by reduction of *p*-hydroxybutyrophenone (Sandulesco and Girard 1930) with aluminium amalgam in moist ether. It crystallizes from alcohol and has m.p. 186–187° (Found: C, 72.4; H, 7.9.  $C_{20}H_{26}O_4$  requires C, 72.7; H, 7.9 %).

The *diacetate*, crystallized from alcohol, has m.p. 198–199° (Found: C, 69.6; H, 7.2.  $C_{24}H_{30}O_6$  requires C, 69.5; H, 7.3 %).

*4:4'-Diacetoxy- $\delta$ : $\epsilon$ -diphenyl- $\gamma$ : $\epsilon$ -octadiene.* The above pinacol was dehydrated by boiling with acetyl chloride and acetic anhydride. The resulting

diacetate, crystallized from alcohol, has m.p. 129–130° (Found: C, 76.3; H, 7.1.  $C_{24}H_{26}O_4$  requires C, 76.15; H, 6.9 %).

4:4'-Dihydroxy- $\delta$ : $\epsilon$ -diphenyl- $\gamma$ : $\epsilon$ -octadiene, prepared by hydrolysis of the diacetate, crystallized from benzene in needles, m.p. 127–128° (Found: C, 81.4; H, 7.3.  $C_{20}H_{22}O_2$  requires C, 81.6; H, 7.5 %).

4:4'-Dihydroxy- $\beta$ : $\gamma$ -diphenyl- $\alpha$ : $\gamma$ -butadiene was prepared in a similar manner from *p*-hydroxyacetophenone pinacol, and crystallizes from benzene in needles, m.p. 164–165° (Found: C, 80.5; H, 5.9.  $C_{16}H_{14}O_2$  requires C, 80.6; H, 5.9 %).

The diacetate, crystallized from alcohol and melted at 118–119° (Found: C, 74.2; H, 5.8.  $C_{20}H_{18}O_4$  requires C, 74.5; H, 5.6 %).

4:4'- $\alpha$ : $\beta$ -Tetrahydroxy- $\alpha\beta$ -dibenzyl- $\alpha\beta$ -diphenylethane was made by reduction of *p*-hydroxyphenyl benzyl ketone with aluminium amalgam in moist ethereal solution and it crystallized from dilute alcohol in needles, m.p. 197–198° (Found: C, 78.5; H, 6.2.  $C_{28}H_{26}O_4$  requires C, 78.8; H, 6.15 %).

The diacetate crystallized from ethyl acetate in small prisms, m.p. 208–209° (Found: C, 75.4; H, 6.0.  $C_{32}H_{30}O_6$  requires C, 75.3; H, 5.9 %).

$\beta$ : $\delta$ -Di(4-hydroxyphenyl)- $\alpha$ : $\delta$ -diphenyl- $\alpha$ : $\gamma$ -butadiene. The diacetate of this substance is prepared by dehydration and simultaneous acetylation of the above pinacol by boiling with acetyl chloride and acetic anhydride. It crystallizes from ethyl acetate and melts at 202° (Found: C, 80.9; H, 5.6.  $C_{32}H_{26}O_4$  requires C, 81.0; H, 5.5 %).

The free dihydroxy-compound crystallizes in needles from alcohol and has m.p. 231–232° (Found: C, 85.9; H, 5.7.  $C_{28}H_{22}O_2$  requires C, 86.1; H, 5.7 %).

$\beta\beta$ -Di(4-hydroxyphenyl)- $\alpha$ -phenylethylene.  $\beta\beta$ -Dianisyl- $\alpha$ -phenylethylene (2.0 g.) (Orechow 1919) was heated in a sealed tube to 190° during 18 hr. with potassium hydroxide (4.0 g.) and alcohol (8 c.c.). On acidification, the solution gave a gummy precipitate which gradually solidified and then crystallized from benzene as pale yellow prisms, m.p. 178° (Found: C, 83.2; H, 5.5.  $C_{20}H_{16}O_2$  requires C, 83.3; H, 5.6 %).

*m*-Methoxyacetophenone. The method of Wahl and Silberzweig (1903, 1905) was found to be tedious and to give very poor yields. A method analogous to that employed for the preparation of acetopiperone (Richardson, Robinson and Seijo 1937) gave an overall yield of 90–95 %, starting from *m*-methoxybenzaldehyde.

4:3'-Dimethoxychalkone. A solution of *m*-methoxyacetophenone (50 g.) and anisaldehyde (45.4 g.) in alcohol (150 c.c.) was shaken for a few minutes with aqueous sodium hydroxide (50 c.c. of 10 %), seeded and kept

overnight at 0°. The yellow crystals were collected, washed well with water and a little alcohol, and recrystallized from dilute alcohol forming yellow prisms, m.p. 52° (55 g.) (Found: C, 76.2; H, 6.0.  $C_{17}H_{18}O_3$  requires C, 76.1; H, 6.0 %).

*γ-Keto-α-cyano-α-anisyl-γ-(m-methoxyphenyl)-propane.* A solution of the chalkone (53.6 g.) in methyl alcohol (150 c.c.) was heated to boiling and sodium cyanide (29.4 g.) added, together with three drops of a solution of phenolphthalein. The mixture was boiled for 20 min., a solution of acetic acid (12 g.) in water (10 c.c.) being added dropwise at such a rate that the reaction mixture remained faintly pink in colour. The formation of a brown colour indicated the end of the reaction. The solution was added to broken ice, the supernatant liquid was decanted from the oil which was washed by decantation and dissolved in hot acetone. On cooling colourless prisms separated, m.p. 92–94° (50 g.). Further recrystallization raised the m.p. to 96–97° (Found: C, 73.1; H, 5.7.  $C_{18}H_{17}O_3N$  requires C, 73.2; H, 5.8 %).

*β-(m-Methoxybenzoyl)-α-anisylpropionamide.* The nitrile (45 g.) was suspended in glacial acetic acid (225 c.c.) and concentrated sulphuric acid (45 c.c.) added slowly with constant shaking. After keeping for 15 min., the mixture was poured on ice. The sticky reddish mass which separated was extracted with chloroform, the combined extracts washed well with water and dried with sodium sulphate. On evaporation, a reddish-brown oil remained which solidified on trituration with alcohol. It crystallized from alcohol in colourless needles, m.p. 112–115° (41 g.). Recrystallized twice more, it melted at 136–137° (Found: C, 68.8; H, 6.2.  $C_{18}H_{19}O_4N$  requires C, 69.0; H, 6.1 %).

*β-(m-Methoxybenzoyl)-α-anisylpropionic acid.* A solution of the amide (35 g.) in alcohol (100 c.c.) was boiled for 10 hr. with aqueous sodium hydroxide (350 c.c. of 7 %). The solution was diluted with water, filtered, and acidified with dilute hydrochloric acid when a brown oil separated and soon solidified. It was collected, dried, and crystallized from ethyl acetate; colourless needles, m.p. 144–147° (30.5 g.). After two further recrystallizations, m.p. 161–162° (Found: C, 68.8; H, 5.7.  $C_{18}H_{18}O_5$  requires C, 68.8; H, 5.7 %).

*γ-(m-Methoxyphenyl)-α-anisylbutyric acid.* A mixture of the keto-acid (28 g.), toluene (125 c.c.), concentrated hydrochloric acid (150 c.c.), aqueous acetic acid (75 c.c. of 5 %) and amalgamated zinc (75 g.) was boiled for 36 hr., with the addition of concentrated hydrochloric acid (50 c.c.) every 9 hr. After cooling, the toluene layer was separated, washed with water and extracted with aqueous sodium carbonate. The combined extracts were acidified, when a milky oil separated which could not be crystallized. It was

extracted with chloroform; on removal of the solvent a yellowish oil remained which was dissolved in hot light petroleum and crystallized in colourless needles on cooling. The m.p. was 95–97°, and after two more recrystallizations, 98–99° (Found: C, 69.1; H, 6.7.  $C_{19}H_{22}O_5$  requires C, 69.1; H, 6.7 %).

*1-Keto-6-methoxy-2-anisyl-1:2:3:4-tetrahydronaphthalene.* The reduced acid (22 g.) was boiled with phosphoryl chloride (75 c.c.) for 5 min., cooled, and poured on ice. The solid which separated was extracted with chloroform and the extracts washed with aqueous sodium carbonate, dried, and evaporated. The brown solid so obtained crystallized from methyl alcohol or ethyl acetate in prisms, m.p. 124–126°; recrystallized again, m.p. 126–127° (15 g.) (Found: C, 76.5; H, 6.4.  $C_{18}H_{18}O_3$  requires C, 76.6; H, 6.4 %). From the sodium carbonate washings, 3.5 g. of unchanged acid were recovered.

*1-Ethyl-2-(p-methoxyphenyl)-6-methoxy-3:4-dihydronaphthalene.* The ketone (14 g.) was added slowly with stirring to a Grignard solution from magnesium (7.7 g.) and ethyl bromide (27.2 g.) in ether (250 c.c.). The mixture was heated for 2 hr. on the steam-bath, cooled, and decomposed with ice and dilute sulphuric acid. The ethereal layer after washing, drying, and evaporation, left an oil which solidified on trituration with alcohol. It was crystallized twice from ethyl acetate; plates, m.p. 94–95° (10.5 g.) (Found: C, 81.2; H, 7.5.  $C_{20}H_{22}O_2$  requires C, 81.6; H, 7.5 %).

*1-Ethyl-2-(p-hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene.* The above dimethoxy-compound (4 g.), potassium hydroxide (20 g.) and alcohol (100 c.c.) were heated together at 165° for 36 hr. in a rotating autoclave. The contents of the autoclave were diluted, and the alcohol removed *in vacuo*. After cooling, the solution was extracted with ether, filtered, heated to remove the ether, cooled, and acidified with dilute hydrochloric acid. The finely divided brown precipitate was taken up in ether and, after removal of the solvent, was washed with ethylene dichloride (10 c.c.) and recrystallized from a mixture of chloroform, ethyl acetate and light petroleum. After repeated recrystallization, the product was obtained as small colourless prisms, m.p. 256° after sintering at 225° (0.5 g.). It turned reddish brown on standing. It was not unsaturated and analysis showed it to be the tetrahydro-compound, disproportionation having apparently occurred (Found: C, 80.1; H, 7.5.  $C_{18}H_{20}O_2$  requires C, 80.5; H, 7.4 %).

The *dibenzoate* crystallized from benzene in plates, m.p. 213–215° (Found: C, 80.0; H, 5.9.  $C_{32}H_{28}O_4$  requires C, 80.6; H, 5.9 %).

*5:14-Dihydroxy-1:2:9:10:11:18-hexahydrochrysene-a.* Dimethoxy-hexahydrochrysene-a (Ramage and Robinson 1933) was dissolved in the

minimum of boiling acetic acid and an equal volume of hydriodic acid (*d.* 1.9) added in the course of 2 min. A new substance soon separated from the clear solution and after boiling for 15 min. the mixture was diluted with water containing sulphur dioxide. The nearly colourless needles were collected, dissolved in cold aqueous sodium hydroxide solution, recovered after filtration by acidification and the solid crystallized from methyl alcohol and then ethyl alcohol and finally from chloroform. The colourless prisms, m.p. 263–264° were very sparingly soluble in alcohol or chloroform (Found: C, 79.9; H, 6.8. After drying at 105° in a high vacuum: C, 80.9; H, 6.7.  $C_{18}H_{18}O_2$  requires C, 81.2; H, 6.7 %).

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## Cytochrome and cytochrome oxidase

BY D. KEILIN, F.R.S., AND E. F. HARTREE, PH.D.

*From the Molleno Institute, University of Cambridge*

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[Plate 1]

### I. INTRODUCTION

It was previously shown that the addition of cytochrome *c* to a heart-muscle preparation greatly increases its power of catalysing the oxidation of such substances as *p*-phenylene diamine, hydroquinone, cysteine and ascorbic acid. It was demonstrated that the oxidations of these substances, which can be used for the detection and estimation of intracellular oxidase, are catalysed not directly by the oxidase but through the co-operation of cytochrome. The only direct function of the oxidase, so far ascertained, is the oxidation of reduced cytochrome, and the enzyme can therefore be considered as cytochrome oxidase (Keilin and Hartree 1938*a*). Several properties which have been previously ascribed to it do not belong, however, to this enzyme alone but are the properties of the complete cytochrome-oxidase system.

The object of this paper is the study of the mechanism of oxidation and reduction of cytochrome in order to determine the properties and the nature of cytochrome oxidase.

### II. HEART-MUSCLE PREPARATION AND SOME OF ITS PROPERTIES

The best material for this study is the heart-muscle preparation obtained by the method described in a previous paper (Keilin and Hartree 1938*a*). This preparation is free from haemoglobin but contains several enzymes

and carriers which are either insoluble or intimately bound to insoluble material.

On spectroscopic examination it shows five diffuse bands in the visible region: (1) a narrow band at about  $605\text{ m}\mu$  which belongs probably to a small fraction of cytochrome *a* in the reduced state, (2) a band at  $567\text{ m}\mu$  which corresponds to the fused  $\alpha$ -bands of oxidized cytochrome components *b* and *c*; (3) a band at  $529\text{ m}\mu$  which represents the fused  $\beta$ -bands of the same two components; and finally (4) and (5) two bands at 495 and  $455\text{ m}\mu$  which belong to a flavoprotein compound (VII, fig. 2, Plate 1).\*

The appearance of this absorption spectrum does not change even when the suspension is kept in pure nitrogen or when treated with cyanide which shows that it is completely devoid of reducing substances and metabolites. This is, moreover, corroborated by the facts that this preparation has no oxygen uptake and does not reduce methylene blue anerobically.

On addition of sodium succinate to this preparation cytochrome becomes reduced, and on shaking the mixture with air it becomes rapidly re-oxidized. Tested manometrically, the suspension now shows a rapid oxygen uptake, which is greatly accelerated by the addition of cytochrome *c* ( $10^{-3}$  to  $10^{-4}\text{ M}$ ), and when tested anaerobically it rapidly reduces methylene blue.

Although this muscle preparation contains other enzymes, including a fairly strong catalase, the only reactions which take place on addition of succinic acid are: (1) its oxidation to fumaric acid, and (2) the formation of malic from fumaric acid catalysed by fumarase; both reactions being reversible.

Other enzymes are practically inactive owing to the absence of their specific substrates and of coenzymes essential for their activities.

### III. ABSORPTION SPECTRUM OF CYTOCHROME IN HEART-MUSCLE PREPARATION

Spectroscopic examination of the reduced cytochrome in this preparation shows a remarkably strong band *a* which is more intense than the bands *b* and *c*. There is an obvious deficiency of cytochrome *c*, as its band, instead of being stronger than that of the component *b*, is of approximately the same intensity.

In addition to the bands *a*, *b* and *c*, which are the  $\alpha$ -bands of the corre-

\* These bands are hardly perceptible in preparations showing reduced cytochrome which may be due either to the reduction of flavoprotein itself or to the changes in the background taking place during the reduction of cytochrome.



sponding components of cytochrome, and the band *d* representing the  $\beta$ -bands of at least the components *b* and *c*, our preparation also shows the three Soret or  $\gamma$ -bands of cytochrome (fig. 1). These bands can easily be seen in a dilute muscle preparation suspended in a 70 % solution of cane sugar or in glycerine and examined with a strong light passing through a suitable colour filter, such as Wratten *C* and *D* filters or a solution of ammoniacal copper sulphate. Bands  $\gamma$  can also be made clearly visible by peptizing muscle preparations with sodium taurocholate or desoxycholate which make the preparation transparent and suitable for spectroscopic examination.\*

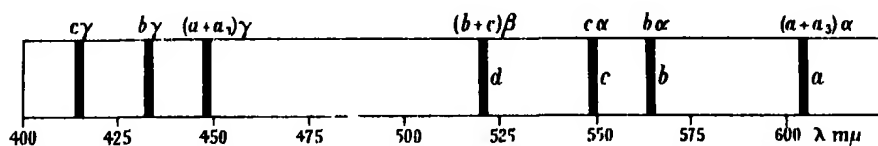


FIG. 1. Diagram showing positions of absorption bands of reduced cytochrome components together with old and new notation of bands.

The  $\gamma$ , like the other bands of cytochrome, can be greatly intensified and sharpened when the suspension is cooled to the temperature of liquid air. For this purpose a very dilute suspension of muscle preparation reduced with sodium succinate is poured into the flattened end of a tube where it forms a layer 2 mm. thick. The tube is cooled in liquid air and rapidly examined spectroscopically.

The  $\gamma$ -bands appear in approximately the same positions as the corresponding bands in the transparent thoracic muscles of bees as described by Warburg and Negelein (1931), namely, 448, 432 and 415  $m\mu$  (fig. 1 and I, fig. 2, Plate 1). That the latter two  $\gamma$ -bands belong respectively to the components *b* and *c* of cytochrome we are able to prove now by showing that each of these bands always appears and disappears simultaneously with the corresponding  $\alpha$ -band and independently of other bands. The true nature of the first  $\gamma$ -band (448  $m\mu$ ) will be discussed later.

It is important to remember that the  $\gamma$ -bands in our preparation are only visible when cytochrome is reduced. In the oxidized state these bands are invisible, the first two  $\gamma$ -bands being probably very weak and masked by general absorption in this region, while that of *c* is shifted towards the blue end of the spectrum (405  $m\mu$ ) and lies outside the region suitable for direct spectroscopic observation.

\* The use of bile salts, which is extensively applied to the study of visual purple, was recommended to us by Dr E. L. Smith.

## IV. EFFECTS OF RESPIRATORY INHIBITORS ON CYTOCHROME

We shall now examine the changes in the absorption spectrum of cytochrome brought about by the addition of substances like CO, KCN,  $\text{NaN}_3$ ,  $\text{H}_2\text{S}$ ,  $\text{NH}_4\text{OH}$ , NaF and peroxides which are known as reversible inhibitors of cellular respiration, or as substances combining reversibly with such haematin compounds as haemoglobin and methaemoglobin, peroxidase and catalase. Throughout this study, unless otherwise stated, cytochrome in the heart-muscle preparation was reduced with sodium succinate. In the description which follows heart-muscle preparation showing the absorption spectrum of reduced cytochrome will be referred to as "reduced cytochrome", the absorption bands as "bands", and oxidized and reduced components of cytochrome will be designated by the corresponding letters and signs of valency, writing, for instance,  $c'''$  and  $c''$  instead of oxidized and reduced components  $c$  of cytochrome. A convenient notation for the absorption bands of cytochrome which is proposed in the present paper (see Table I, p. 177) consists in the customary use of the letters  $\alpha$ ,  $\beta$  and  $\gamma$  to denote the three bands of each component together with prefixes  $a$ ,  $b$  and  $c$  to differentiate between the cytochrome components. Thus, the  $\alpha$ -,  $\beta$ - and  $\gamma$ -bands of the component  $c$  will be respectively referred to as  $c\alpha$ ,  $c\beta$  and  $c\gamma$ .

We can mention at this stage that a critical analysis of the experimental results now to be described has led us to the conclusion that there exists in aerobic cells a new cytochrome component which we have denoted as  $a_3$  (Keilin and Hartree, 1938*b*).

## DESCRIPTION OF PLATE I

FIG. 2. Diagrammatic figure of reconstructed absorption spectra of reduced and oxidized cytochrome in heart-muscle preparation, untreated and treated with different respiratory inhibitors. Bands of each spectrum are represented as seen in three different depths of preparation; their ratio, according to bands, being approximately as follows: 8 for  $\alpha$  and  $\beta$  bands; 2.5 for  $a\gamma$  and  $a_3\gamma$ ; 1.5 for  $b\gamma$  and  $c\gamma$ . Band  $c\gamma$  is seen more distinctly because deficiency of this component in this preparation is compensated by addition of soluble  $c$ . Notation of bands given above and below diagram refers only to spectra I and VII respectively. Spectrum II shows  $a_3''$  CO  $\alpha$ -band at  $590\text{ m}\mu$ , while band  $a_3'$  CO  $\gamma$  appears at  $432\text{ m}\mu$ , where it overlaps and intensifies band  $b\gamma$ . Band  $a\gamma$  is seen at  $452\text{ m}\mu$ . In spectrum III bands  $\alpha$  and  $\gamma$  of  $a_3$  are invisible owing to its oxidation and combination with inhibitors. Spectra IV, V and VI are clearly explained in the figure. Spectrum VII, representing oxidized cytochrome reconstructed from different depths of preparation, shows remains of reduced  $\alpha\alpha$ -band, two diffuse para-haematin bands ( $p\alpha$  and  $p\beta$ ) of compounds  $b$  and  $c$ , and two diffuse bands ( $f$ ) of a flavoprotein compound, which become hardly perceptible in preparations showing reduced cytochrome.

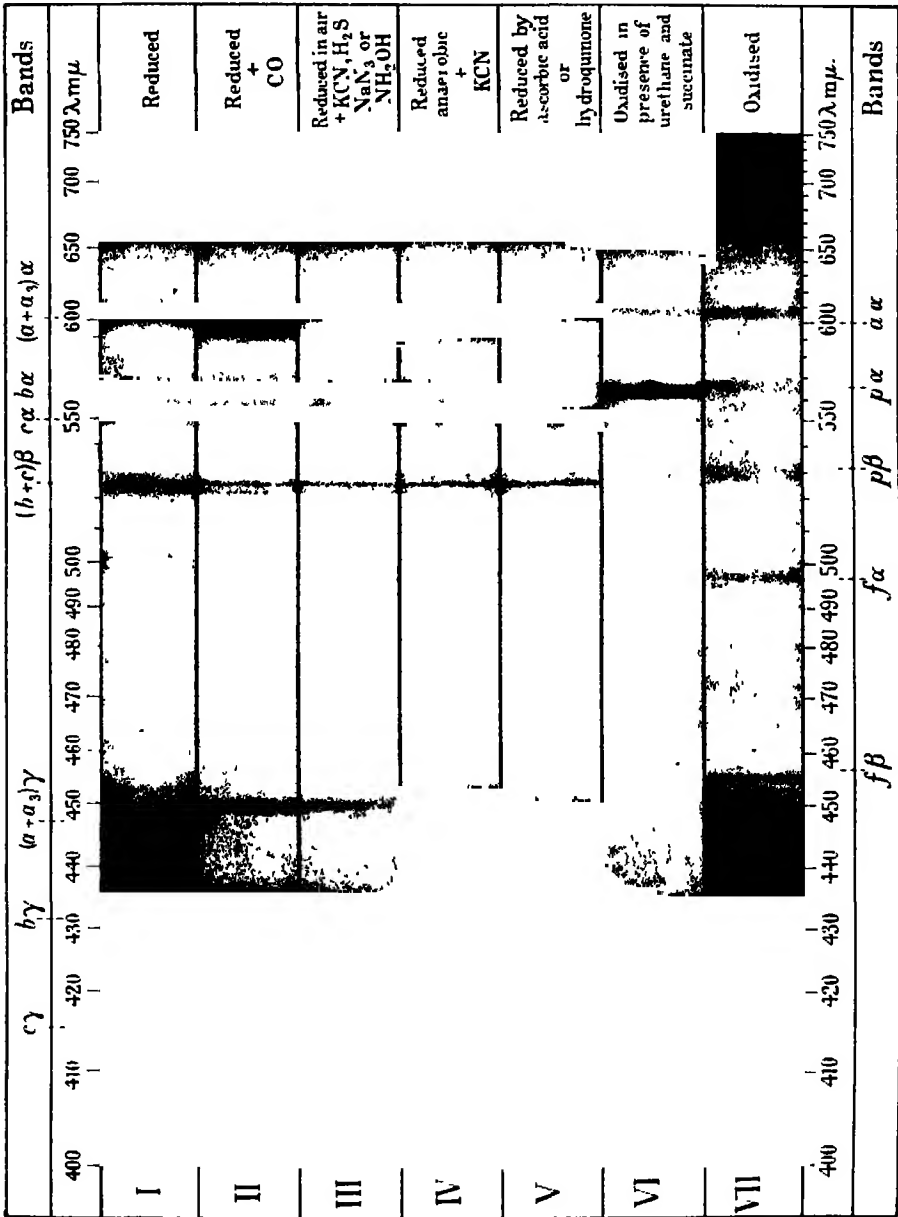


FIG. 2



(1) *Effect of CO*

On treating cytochrome with carbon monoxide, the bands of  $b\alpha$ ,  $c\alpha$  and  $(b + c) \beta$  remain unmodified, while the band  $a\alpha$  undergoes a very marked change. It becomes wide, asymmetric and spreads towards the blue end of the spectrum, partly obliterating the space which separates it from the band  $b\alpha$ . It shows, moreover, an additional reinforcement at about  $590 m\mu$ , while its long wave side remains sharply defined. The strong band  $a\gamma$  ( $448 m\mu$ ) disappears and is replaced by a very pale band at  $452 m\mu$ , while  $b\gamma$  becomes much stronger and sharper (II, fig. 2, Plate 1). The correct interpretation of these changes can only be arrived at by studying the effect of other inhibitors on cytochrome.

(2) *Effect of KCN*

The effect of KCN on reduced cytochrome can be studied in two ways: *aerobically* in open tubes and *anaerobically* in Thunberg tubes.

In *aerobic* experiments, a small amount of cyanide is added to the muscle preparation containing sodium succinate and showing reduced cytochrome. Spectroscopic examination of the mixture does not reveal any marked change in the  $\alpha$ -bands of all the components. On the other hand,  $a\gamma$  disappears and is replaced by a weak band at  $452 m\mu$ , while  $b\gamma$  and  $c\gamma$  remain unchanged (III, fig. 2, Plate 1). On treating this preparation with CO no changes can be noticed in the spectrum. This shows that KCN, which affects only the band  $a\gamma$ , prevents the formation of the CO compound which is so easily formed in absence of KCN. Moreover, on addition of KCN to the preparation treated previously with CO, the spectrum due to the presence of CO disappears and is replaced by that which is obtained in presence of KCN.

In *anaerobic* experiments, the muscle preparation (4 c.c.) containing sodium succinate and a drop of caprylic alcohol is put into a modified Thunberg tube the hollow stopper of which receives 0.5 mg. of solid KCN. The tube is evacuated and, after standing for 15 min., its contents are mixed. Spectroscopic examination now reveals a very marked change in the appearance of band  $a\alpha$  which broadens towards the blue end of the spectrum and has a distinct appearance of being double. Band  $a\gamma$  becomes wider and somewhat more diffuse lying now at  $450 m\mu$ , while the other bands remain unchanged (IV, fig. 2, Plate 1). On letting air in, the appearance of the spectrum changes at once and reverts to that of reduced cytochrome treated with KCN in an open tube. Similar results can be obtained also in an open tube provided cytochrome is reduced not by the succinic system but by an excess of  $\text{Na}_2\text{S}_2\text{O}_4$ .

No clear changes can be observed on addition of KCN to oxidized cytochrome which is due to the diffuse appearance of its spectrum.

### (3) *Effects of $H_2S$ , $NaN_3$ and $NH_2OH$*

These substances act on cytochrome in the same way as KCN but only under aerobic conditions. When added to reduced cytochrome a marked change is seen only in the band  $\alpha\gamma$  which is replaced by a weak and diffuse band at  $452 m\mu$ , while other bands remain unmodified (III, fig. 2, Plate 1). Like KCN these substances prevent the formation of a compound with CO. This inhibition is, however, not as complete as that produced by KCN. In fact, even if allowed to stand in presence of an excess of these substances, the CO compound is gradually formed.

### (4) *Effect of NaF*

Sodium fluoride cannot be considered as an inhibitor of the oxidase. On the contrary, in presence of sodium succinate, it inhibits not the oxidation but the reduction of cytochrome. This inhibition, as can be demonstrated spectroscopically and manometrically, is not very marked. Thus, NaF in concentrations of 0.003, 0.01 and 0.02 M inhibits the oxygen uptake by only 15, 42 and 70 % respectively. On treating the preparation with NaF the  $\alpha\gamma$ -band ( $448 m\mu$ ) is replaced by a weak band lying at  $452 m\mu$ , while other bands of cytochrome remain unchanged. On standing, this band very soon reverts to its usual intensity and position which shows that the effect of NaF on cytochrome is not as marked as that of  $NaN_3$  or  $NH_2OH$ .

### (5) *Effect of peroxides*

The effect of hydrogen peroxide on cytochrome in heart-muscle preparation is very difficult if not impossible to observe owing to the presence in our preparation of a sufficiently active catalase to decompose rapidly all the  $H_2O_2$  added to it. The addition of cyanide or of hydroxylamine to poison the catalase does not help in this case because these inhibitors, as has just been shown, also react with cytochrome. Although we have failed so far to discover a reaction between cytochrome and  $H_2O_2$  the experiments with hydrogen peroxide were not devoid of interest. They have shown, for instance, that a great excess of  $H_2O_2$  added to the muscle preparation containing sodium succinate and 0.003 M cyanide did not oxidize the reduced components  $a$ ,  $b$  and  $c$ . This seems to indicate that if

$\text{H}_2\text{O}_2$  is formed within living cells as a result of some primary oxidation reactions, it would not oxidize reduced cytochrome. The previously observed oxidation of extracted cytochrome *c* by  $\text{H}_2\text{O}_2$  may be due to a peroxidatic reaction catalysed either by *c* itself or by some haematin derivatives resulting from their partial destruction by  $\text{H}_2\text{O}_2$ . It is also possible that all the components of cytochrome in heart-muscle preparation, including *c*, are absorbed to, or combined with, some insoluble material, which protects them from oxidation and destruction by  $\text{H}_2\text{O}_2$ .

In order to observe an effect of a peroxide on cytochrome an attempt was made to replace  $\text{H}_2\text{O}_2$  by ethylhydroperoxide which is not decomposed by catalase. A slight complication in these experiments is the partial oxidation of cytochrome. The experiments properly controlled seem, however, to show that the addition of ethylhydroperoxide causes the fading of the band  $\alpha\gamma$  and is responsible for the delay in the formation of a compound with carbon monoxide. In other words the effect of ethylhydroperoxide on cytochrome is somewhat similar to, although less marked than, that of KCN,  $\text{NaN}_3$  or  $\text{NH}_2\text{OH}$ .

## V. EXISTENCE OF COMPONENT $a_3$

The experiments related in the previous chapter show that the absorption spectrum of reduced cytochrome in the heart-muscle preparation is affected by substances like CO, KCN,  $\text{NaN}_3$ ,  $\text{H}_2\text{S}$ ,  $\text{NH}_2\text{OH}$ , NaF and  $\text{C}_2\text{H}_5\text{OOH}$  which are known either as respiratory inhibitors or as substances combining with different haematin compounds. According to the effects which they produce on cytochrome these substances can be grouped into three categories:

(1) CO which affects the bands  $\alpha\alpha$ ,  $\alpha\gamma$  and  $b\gamma$  and modifies them in a similar manner whether the reaction takes place anaerobically or in presence of a small concentration of oxygen.

(2) KCN which affects the bands  $\alpha\alpha$  and  $\alpha\gamma$  but only under strictly anaerobic conditions.

(3) KCN,  $\text{NaN}_3$ ,  $\text{H}_2\text{S}$ , NaF,  $\text{NH}_2\text{OH}$  and  $\text{C}_2\text{H}_5\text{OOH}$  which markedly affect only the band  $\alpha\gamma$  under aerobic conditions; the presence of oxygen being essential for this reaction.

In all these experiments only the bands  $\alpha\alpha$ ,  $\alpha\gamma$  and  $b\gamma$  of cytochrome appear to be affected by the respiratory inhibitors. At first sight these experiments seem to suggest that the changes observed in the absorption spectra of cytochrome are simply due to the formation of compounds

between the components *a* or *b* and the substances mentioned above. Careful analysis of these results, however, does not support this view. In fact, it is difficult to understand why the formation of a compound between *a* and KCN or  $\text{NaN}_3$ , for instance (III, fig. 2, Plate 1), requires the presence of oxygen and affects only the band  $a\gamma$ , while the band  $a\alpha$ , being unchanged, clearly shows that component *a* remains still in the reduced state. We can hardly ascribe these changes to the combination of *a* with KCN without postulating the formation of a peculiar compound, one portion of which, responsible for band  $a\alpha$ , is reduced, while the other, responsible for band  $a\gamma$ , is oxidized. The existence of such a compound is, moreover, difficult to reconcile with the formation of another KCN compound which only takes place under strictly anaerobic conditions (IV, fig. 2). Finally, it is difficult to explain how CO can affect simultaneously both  $\alpha$ - and  $\gamma$ -bands of one component (*a*) and only the  $\gamma$ -band of another component (*b*) (II, fig. 2).

These facts make it very difficult if not impossible to accept the view that the observed spectroscopic changes are due to the formation of compounds between the components *a* or *b* and the respiratory inhibitors.

The only satisfactory explanation of these changes which takes into account the results of all our experiments consists in postulating the existence of a new component, the absorption bands of which have not been hitherto recognized because they lie too closely to the corresponding bands of component *a*. We shall designate this new component as  $a_3$  in order to distinguish it from components  $a_1$  (589  $m\mu$ ) and  $a_2$  (630  $m\mu$ ) which are known to occur only in a few bacteria devoid of component *a*.<sup>\*</sup> The absorption bands  $a\alpha$  and  $a\gamma$ , which have hitherto been considered as belonging to component *a* only, are in fact the fused bands of two components *a* and  $a_3$ . While the band  $a_3\alpha$  is weak compared with  $a\alpha$  and lies probably at about 600  $m\mu$ , the band  $a_3\gamma$ , lying at 448  $m\mu$ , is, on the contrary, much stronger than  $a\gamma$ , which occupies only the long wave margin of  $a_3\gamma$  and lies at 452  $m\mu$  (fig. 3).

If the component  $a_3$  now undergoes oxidation, while the component *a* remains reduced, the band  $\alpha$  (605  $m\mu$ ) will hardly change its intensity but the band  $\gamma$  (448  $m\mu$ ) will disappear leaving a pale shading at 452  $m\mu$ . The component  $a_3$  differs, moreover, from *a* in several other properties. Thus while *a* is not autooxidizable and does not react with respiratory inhibitors,  $a_3$  is autooxidizable and forms with these inhibitors definite and more or less stable compounds.

<sup>\*</sup> The nature of these two components ( $a_1$  and  $a_2$ ), which have certain properties in common with  $a_3$ , and their relationship with the latter, will be discussed in a separate paper.



With CO, the component  $a_3$  combines only in the divalent state forming a compound with two bands shifted towards the blue end of the spectrum. Thus, the  $a_3\alpha$ -band becomes distinctly visible lying at about 590  $m\mu$  and remaining still fused with and broadening the  $\alpha\alpha$ -band. The  $a_3\gamma$ -band moves from 448 to 432  $m\mu$ , where it overlaps and intensifies the  $b\gamma$ -band (II, fig. 2 and fig. 3). The weak  $a\gamma$ -band becoming thus unmasked can be seen at about 452  $m\mu$ .

With KCN  $a_3$  combines both in the divalent and trivalent states, forming two distinct compounds. The former compound, which can only be obtained under strictly anaerobic conditions, shows a very characteristic absorption spectrum (IV, fig. 2, Plate 1). This derivative is, however, unstable and easily undergoes autoxidation to a trivalent  $a_3$  CN compound.

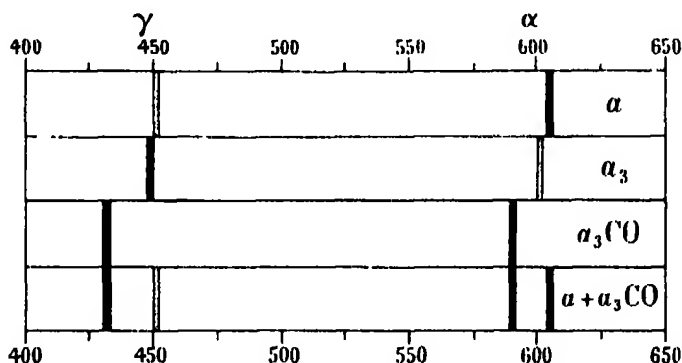


FIG. 3. Diagram showing relationship between bands of components  $a$  and  $a_3$  and effect of CO on  $a_3$ .

In the trivalent state  $a_3$  has a very great affinity for KCN. When reduced cytochrome exposed to air is treated with cyanide,  $a_3$  undergoes oxidation and readily combines with KCN forming a compound ( $a_3^{+++}$  CN) which cannot be easily reduced by the succinic system. Cyanide can be considered therefore as an inhibitor stabilizing this component in the oxidized state. The great affinity of  $a_3^{+++}$  for cyanide and the stability of the  $a_3^{+++}$  CN compound explain the remarkable property of  $a_3$  to undergo oxidation on the addition of KCN, even in the presence of only traces of oxygen, when all the other components of cytochrome are completely reduced. It must be remembered that trivalent  $a_3$  whether free or combined with cyanide does not show distinct absorption bands in any region of the visible spectrum. The formation of the  $a_3^{+++}$  CN compound must therefore be accompanied by the disappearance of the  $a_3\alpha$ - and  $a_3\gamma$ -bands. The disappearance of the  $a_3\alpha$ -band would hardly affect the intensity of the band

$\alpha\alpha$  of which it represents only a small fraction. On the contrary, the disappearance of the  $a_3\gamma$ -band reduces the strong  $\gamma$ -band ( $448\text{ m}\mu$ ) to a very weak band lying at  $452\text{ m}\mu$  and belonging to the component  $a$ . The existence of  $a_3$  clearly explains the changes in the absorption spectrum of reduced cytochrome taking place in presence of KCN and traces of oxygen.

Other substances like  $\text{H}_2\text{S}$ ,  $\text{NaN}_3$ ,  $\text{NH}_2\text{OH}$  and  $\text{NaF}$  combine only with trivalent  $a_3$  forming with it a series of compounds analogous to  $a_3^{\text{III}}\text{CN}$ . The affinities of  $\text{H}_2\text{S}$  and  $\text{NaN}_3$  for  $a_3$  are, however, somewhat lower than that of KCN but much higher than those of  $\text{NH}_2\text{OH}$  and  $\text{NaF}$ . The compounds of  $a_3$  with the latter two substances are unstable, and on standing in presence of a reducing system rapidly undergo dissociation followed by the reduction of  $a_3$  as indicated by the reappearance of the band  $a_3\gamma$  ( $448\text{ m}\mu$ ).

Although normally the components  $a$  and  $a_3$  are found in the same state of valency, conditions can be realized when one of the components is oxidized while the other is reduced. Thus, in presence of inhibitors like KCN,  $\text{H}_2\text{S}$ ,  $\text{NaN}_3$ , etc., and traces of oxygen, the component  $a_3$ , as we have seen, undergoes oxidation and combines with these inhibitors while component  $a$  remains reduced. On the other hand, on shaking the preparation containing CO with air, the component  $a$  may undergo oxidation while  $a_3$  remains almost completely reduced and combined with CO. All this strongly supports the view that we are dealing here with two distinct but closely allied and intimately connected compounds  $a$  and  $a_3$ .

## VI. PROPERTIES OF CYTOCHROME COMPONENTS

Before discussing the possible functional relationships between  $a_3$  and the other components of cytochrome we shall briefly record their properties. The positions of the bands of these components together with those of the two spectroscopically recognizable derivatives of  $a_3$  are given in Table I. This table also shows the new notation for the absorption bands proposed in this paper compared with the notation previously used. It can be mentioned here that so far no  $\beta$ -band could be detected in the components  $a$  and  $a_3$  or in derivatives of the latter. This band is probably masked by the  $\alpha$ -band of components  $b$  or  $c$ .

### (1) Component $a$

The main properties of this component have already been considered in the section dealing with the effects of inhibitors on cytochrome. We may add here that this component is a haem-protein compound which is in-

soluble or intimately bound to the insoluble material of the cell. It is thermolabile, undergoing destruction above 52° C. It does not combine with KCN, CO and other inhibitors, and is very easily reduced by sodium succinate. Its absorption bands are as follows:

$$a\alpha - 605 \text{ m}\mu, \quad a\beta - ?, \quad \text{and} \quad a\gamma - 452 \text{ m}\mu.$$

TABLE I

Components of cytochrome	Notation of bands		Position in $\text{m}\mu$
	Previously used	Proposed now	
<i>a</i>	<i>a</i>	<i>a</i> $\alpha$	605
	—	<i>a</i> $\beta$	?
	—	<i>a</i> $\gamma$	452
<i>a</i> <sub>3</sub>	—	<i>a</i> <sub>3</sub> $\alpha$	600
	—	<i>a</i> <sub>3</sub> $\beta$	?
	—	<i>a</i> <sub>3</sub> $\gamma$	448
<i>b</i>	<i>b</i>	<i>b</i> $\alpha$	564
	<i>d</i>	<i>b</i> $\beta$	530
	—	<i>b</i> $\gamma$	432
<i>c</i>	<i>c</i>	<i>c</i> $\alpha$	550
	<i>d</i>	<i>c</i> $\beta$	521
	—	<i>c</i> $\gamma$	415
<i>a</i> <sub>3</sub> + CO	—	<i>a</i> <sub>3</sub> (CO) $\alpha$	590
	—	<i>a</i> <sub>3</sub> (CO) $\beta$	?
	—	<i>a</i> <sub>3</sub> (CO) $\gamma$	432
<i>a</i> <sub>3</sub> + CN	—	<i>a</i> <sub>3</sub> (CN) $\alpha$	590
	—	<i>a</i> <sub>3</sub> (CN) $\beta$	?
	—	<i>a</i> <sub>3</sub> (CN) $\gamma$	450

### (2) Component *a*<sub>3</sub>\*

This component, as we have seen, is thermolabile and combines with the respiratory inhibitors such as CO, KCN, H<sub>2</sub>S, NaN<sub>3</sub>, NH<sub>2</sub>OH and probably with NaF and C<sub>2</sub>H<sub>5</sub>OOH. Its absorption bands are:

$$a_3\alpha - 600 \text{ m}\mu, \quad a_3\beta - ?, \quad \text{and} \quad a_3\gamma - 448 \text{ m}\mu.$$

\* For reference to components *a*<sub>1</sub> and *a*<sub>2</sub>, see footnote, p. 174.

### (3) *Component b*

This component is also a thermolabile haem-protein compound showing three absorption bands in the reduced states:  $b\alpha$ —564  $m\mu$ ,  $b\beta$ —530  $m\mu$  and  $b\gamma$ —432  $m\mu$ . It is also insoluble or bound to insoluble material of the cell and cannot therefore be isolated in an unmodified or undenatured state. The so-called "extracted cytochrome *b*" of Yakushiji and Mori (1937) is probably only a haematin derivative of *b* mixed with some denatured proteins and cannot therefore be identified with native *b*. Cytochrome *b* differs from components *a* and *c* in being distinctly autoxidizable. In fact, if *b* is reduced by substances like ascorbic acid or adrenaline which do not require to be activated by specific dehydrogenases, it can be reoxidized in air even in presence of a great excess of cyanide. If *b* is reduced by succinic acid, its autoxidation in presence of cyanide can only be observed when the dehydrogenase system is inhibited by the addition of sodium malonate. It is important to remember, however, that cytochrome *b* is much more easily reduced by the succinic dehydrogenase system than by the reducing substances like ascorbic acid, adrenaline, hydroquinone or *p*-phenylenediamine (V, fig. 2, Plate 1). This indicates that component *b* does not play an important role in the catalytic oxidation of these compounds. The integrity of the component *b* in the heart-muscle preparation treated in different ways cannot therefore be recognized from the mere fact that the catalytic oxidation of *p*-phenylenediamine by this preparation proceeds normally.

There are, on the other hand, clear indications that the activity of the component *b* is intimately connected with those of dehydrogenases. The aerobic oxidation of substances like succinic acid depends not only on the integrity of components *a*,  $a_3$  and *c* but also on that of *b*. This is clearly shown by the behaviour of cytochrome *b* in presence of narcotics (Keilin 1925) and can be illustrated by the following experiment: 3 c.c. of heart-muscle preparation, containing a small amount of succinic acid and showing rapid oxidations and reductions of cytochrome, receives 0.3 c.c. of a 30 % solution of urethane. On shaking the suspension with air, components *a*,  $a_3$  and *c* are rapidly oxidized and do not undergo reduction even on standing, while *b* remains completely reduced even on vigorous shaking with air and clearly shows its bands  $b\alpha$ ,  $b\beta$  and  $b\gamma$  (VI, fig. 2, Plate 1). Urethane, therefore, like all other narcotics, inhibits the reduction of *a*,  $a_3$  and *c* and the oxidation of *b*. According to Tamiya and Ogura (1937), of the three components of cytochrome, *b* reacts directly with the dehydrogenase system, *c* with the oxidase and oxygen, while *a* forms a link between

*b* and *c*, being reduced by the former and oxidized by the latter. The peculiar effect of urethane on component *b* they ascribe to its inhibition of the activity of *a*, thus breaking the link between *b* and *c*. Considering that narcotics inhibit to a certain degree the anaerobic reduction of methylene blue by the same systems, the supposition of these workers would naturally imply that this reaction also proceeds through at least two components of cytochrome, *a* and *b*. So far, there is no direct evidence supporting this view. In fact, even in the presence of narcotics and sodium succinate *b* can undergo oxidation, when the activity of succinic dehydrogenase is abolished by the addition of oxalacetic acid. Moreover, when *b* is reduced by substances not activated by dehydrogenases, narcotics do not prevent its reoxidation. It is conceivable that the effect of narcotics consists in bringing about the formation of a not easily dissociable complex composed of dehydrogenase, substrate and cytochrome *b* and so making it inaccessible to the portion of the system reacting with oxygen.

It is important to mention here that narcotics, which hardly affect the oxidation of *p*-phenylenediamine, inhibit more strongly the aerobic oxidation of succinic acid by the cytochrome system than its anaerobic oxidation by methylene blue. It appears, therefore, that there is no strict parallelism between the property of the muscle preparations to reduce cytochrome *c* and their property to reduce methylene blue. In fact, from the heart-muscle suspension treated in different ways, preparations can be obtained which in presence of succinic acid hardly reduce cytochrome *c* and are yet capable of rapidly oxidizing the substrate by means of methylene blue.\* This seems to indicate that the activated molecules of substrate do not react with cytochrome *c* directly but through another and more labile compound which may be either cytochrome *b* itself or a hitherto unrecognized component of the system.

#### (4) Component *c*

Cytochrome *c*, unlike the other components, is thermostable and comparatively resistant to denaturation. It is soluble or perhaps more easily detached from the insoluble material of the cell and can therefore be extracted and very easily prepared in a pure state (Keilin and Hartree 1937). Its spectrum is composed of three bands:  $\alpha\lambda$  550  $m\mu$ ,  $c\beta$ —521  $m\mu$  and  $c\gamma$ —415  $m\mu$ . Within the physiological range of pH it is not autooxidizable and does not combine with CO. In the oxidized state it does not combine with KCN,  $H_2S$  or  $NaN_3$ . It combines very readily, however, with NO.

\* The reduction of cytochrome by the succinic and other dehydrogenase systems will be discussed in a special paper dealing with this subject.

At pH below 4 and especially above 12 it becomes autoxidizable and combines with CO forming a compound very sensitive to light. Oxidized *c* is easily reduced both by activated metabolites and by a great variety of reducing substances such as cysteine, adronaline, ascorbic acid and *p*-phenylenediamine. Reduced *c* is, on the other hand, very rapidly oxidized when brought into contact with even a very dilute preparation of heart muscle.

On applying to the study of cytochrome *c* a greatly improved spectrophotometric technique, Altschul and Hogness (1938) believed to have found that this component combines with CO throughout the entire range of pH. It is, however, difficult to draw such a conclusion from the curves given by these workers, as the slight effect of CO which they reveal at pH 7.96 may be due to its combination either with a small fraction of denatured *c* or with some other haematin impurity in their preparation. That CO does not combine with unmodified cytochrome *c* below pH 13 can be demonstrated by the following manometric experiments.

80 c.c. of strong cytochrome *c* solution obtained from two horse hearts is precipitated by 4 vol. of cold acetone, resuspended in a small volume of water and dialysed against 0.5 % NaCl. A slight precipitate is filtered off giving 20 c.c. of a clear and very strong solution containing 0.0565 mg. of cytochrome Fe per c.c.; 4.4 c.c. of this solution being equivalent to 100 cu. mm. of CO.

The manometric experiments are carried out in Barcroft differential manometers at 19° C. The right-hand flask of one receives 4.4 c.c. cytochrome *c*, 1.6 c.c. 2M phosphate buffer pH 7, and 5 mg. of dry  $\text{Na}_2\text{S}_2\text{O}_4$  in a dangling tube suspended from the central potash tube. The left-hand flask receives 4.4 c.c. water, 1.6 c.c. buffer and 5 mg.  $\text{Na}_2\text{S}_2\text{O}_4$  in a dangling tube. The manometer is evacuated, washed three times with pure  $\text{N}_2$  and filled with pure CO. After temperature equilibration, the dangling tubes are dislodged and the manometer is read every 5 min.

TABLE II. THEORETICAL ABSORPTION, 100 CU. MM.

Exp. no.	pH	cu. mm. CO absorbed
1	7	11
2	10	21
3	13	104

Two other experiments were carried out in a similar way but in more alkaline solutions, one at pH 10 and the other at pH 13. The results of these experiments, which are given in Table II, show that only at pH 13

does cytochrome  $c''$  combine with CO. The small uptakes at lower pH are probably due to a slight denaturation of cytochrome  $c$  by acetone.

A significant contribution to our knowledge of the structure of the cytochrome  $c$  molecule has been made by Theorell (1938) who was able to correlate its stability with a unique double thioether linkage between the protein and the prosthetic group.

## VII. RELATIONSHIP BETWEEN COMPONENTS $a$ AND $a_3$

The invariable association of components  $a$  and  $a_3$  clearly indicates that they are in some way intimately connected. This is, moreover, supported by the existence of a definite proportionality between these two components; namely, the stronger the absorption bands of  $a$  in a preparation the more intense are the bands of spectroscopically visible derivatives of  $a_3$ . In other words  $a_3$  can only be detected easily in cells showing a high concentration of  $a$ .

That these two components have an identical haem nucleus is shown by the similarity of their absorption spectra and, more so, by the fact that on denaturation with alkali and treatment with pyridine both components yield only one spectroscopically recognizable haemochromogen. This haem nucleus is probably very similar to that of chlorocruorin.

The proteins of these compounds also show great similarities in their properties. Thus, both are insoluble or bound to insoluble material of the cell, and both exhibit the same degree of fragility when treated with alkali, acids, alcohols and acetone, dried in air or warmed above 52° C. Both compounds are, if anything, less stable than most enzymes.

On denaturation by any treatment the  $\alpha$ -band of these compounds moves towards the blue end of the spectrum to occupy a position at about 583 m $\mu$ . This derivative (of  $a$  and  $a_3$ ) has the properties of an ordinary haemochromogen, namely, it is autoxidizable and combines with CO. The native proteins of  $a$  and  $a_3$  must, however, differ in some respects so as to form with the same haem nucleus two compounds having distinct properties. The differences may also be of such a nature as not to exclude the interconvertibility of these two components.

## VIII. COMPONENT $a_3$ AND CYTOCHROME OXIDASE

### (1) *Evidence for identity of $a_3$ and oxidase*

As to the physiological significance of  $a_3$ , the evidence which supports the view of its identity with cytochrome oxidase can be summarized as follows:

(a) Like oxidase  $a_3$  is thermolabile being affected by temperatures above  $52^\circ \text{C}$ .

(b) Its oxidations and reductions can be followed spectroscopically within living cells by examination of band  $a_3\gamma$  ( $448 \text{ m}\mu$ ).

(c) It is rapidly and efficiently reduced by biological reducing systems.

(d) It combines reversibly with such substances as KCN,  $\text{H}_2\text{S}$ ,  $\text{NaN}_3$ ,  $\text{NH}_2\text{OH}$  and CO, which are known as reversible inhibitors of oxidase activity and of the respiration of cells.

(e) The two bands of its compound with CO ( $a_3'' \text{CO}$ ) namely,  $\alpha$ — $590 \text{ m}\mu$  and  $\gamma$ — $432 \text{ m}\mu$ , occupy approximately the same positions as the corresponding bands in the photochemical absorption spectrum obtained by Warburg and his co-workers (see Warburg 1932).

(f) Although KCN combines both with divalent and trivalent  $a_3$ , the divalent  $a_3''$  KCN rapidly undergoes autoxidation, while the trivalent  $a_3'''$  KCN is very stable and does not easily undergo reduction. The inhibition of the catalytic properties of this component is therefore due to its stabilization in the ferric state.

(g) The component  $a_3$  can be observed not only in a heart-muscle preparation, but also in fresh thoracic muscles of bees and other insects, in baker's yeast and in aerobic bacteria.

The view which identifies the component  $a_3$  with cytochrome oxidase corroborates, therefore, the results previously obtained by Warburg and his co-workers who have shown that a haematin with a definite absorption spectrum plays an essential role in cellular respiration.

## (2) *Some difficulties in the identification of $a_3$ with cytochrome oxidase*

Although the hypothesis identifying the component  $a_3$  with cytochrome oxidase is supported by strong evidence, several observations have been made in the course of this investigation which do not appear to corroborate this view. Before examining these observations in detail we can say at once that some of the difficulties so raised are only apparent and may readily be explained. On the other hand, certain difficulties which still remain require further investigation before the true nature of  $a_3$  and its relationship to cytochrome oxidase can be definitely established.

### A. *Oxidation of components a, b, and c in presence of $a_3'' \text{CO}$ compound.*

On shaking with air a preparation containing succinic acid and treated with CO the components *a*, *b* and *c* undergo oxidation while the spectrum of  $a_3'' \text{CO}$  remains still clearly visible. This experiment seems to indicate that the components *a*, *b* and *c* could not possibly be oxidized by  $a_3$  since



it remained combined with CO. A careful analysis of this experiment has convinced us now that this objection is only apparent. The absorption spectrum of  $a_3^{''}$  CO which remains visible during the oxidations of the other components indicates only that the rate of reduction of  $a_3$  and formation of  $a_3^{''}$  CO is faster than the rate of its oxidation. If oxidized  $a_3^{'''}$  is capable of oxidizing the three other components, the small amount of  $a_3^{'''}$ , which is being constantly formed under these conditions from the  $a_3^{''}$  CO compound, could easily account for the oxidation of components  $a$ ,  $b$  and  $c$ , even though the bands of  $a_3^{''}$  CO remain clearly visible.

#### B. Functional relationship between $a_3$ and $c$ .

If the component  $a_3$  is the oxidase it should be possible to demonstrate spectroscopically its reaction with at least one of the non-autoxidizable cytochrome components. As cytochrome  $c$  was proved to be essential for the catalytic activity of the oxidase (Keilin and Hartree 1938*a*) a large amount of the divalent  $c^{''}$  added to the muscle preparation should directly or indirectly reduce the component  $a_3^{'''}$ . Experimentally this problem can only be approached by bringing together under strictly anaerobic conditions a heart-muscle preparation and a solution of reduced cytochrome  $c$ , both completely free from reducing substances and metabolites. In order to reduce cytochrome  $c$  for this purpose a strong solution of it in 0.1 M  $\text{Na}_2\text{HPO}_4$  is mixed with platinum black and treated with a current of hydrogen. When cytochrome  $c$  is completely reduced the platinum black is filtered off and the solution kept in a Thunberg tube free from oxygen.

The experiment is carried out in a modified Thunberg tube provided with a hollow stopper and an additional rotating side bulb. The tube receives 3 c.c. of fresh heart-muscle preparation, the hollow stopper receives 0.5 c.c. of reduced  $c$  ( $10^{-3}$  M) and about 1 c.c. of chromous chloride solution is placed in the side bulb. The tube is evacuated, washed out with purified nitrogen, and shaken in order to complete the removal of oxygen. On spectroscopic examination the preparation shows only the faint bands of oxidized cytochrome. On mixing the contents of the tube with that of the hollow stopper, a certain portion of cytochrome  $c$  becomes oxidized by some traces of oxygen still present in the muscle preparation but neither the band  $\alpha\alpha$  nor the band  $\gamma$  of  $a + a_3$  can be seen. The same result was obtained with a dilute preparation of heart muscle suspended in cane sugar and therefore more suitable for the study of the  $\gamma$ -bands. Here also no traces of the band  $\alpha\gamma + a_3\gamma$  could be detected while a portion of  $c$  remained in the

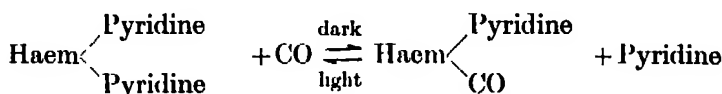
reduced state. These experiments gave therefore no evidence supporting the view that cytochrome  $c''$  can be directly or indirectly oxidized by  $a_3'''$ .

If, on the other hand, the Thunberg tube is evacuated and filled with pure CO the addition of reduced  $c$  to the muscle preparation induces the formation of the  $a_3''$  CO compound. One of the difficulties connected with this experiment is the tendency of  $a_3'''$ , in presence of CO, to undergo gradual reduction with the formation of  $a_3''$  CO even without the addition of the reduced  $c$ . This reduction is, however, accelerated when reduced  $c$  is added to the preparation.

In view of certain technical difficulties connected with such experiments we cannot yet definitely exclude the possibility that divalent  $c''$  may yet be oxidized by trivalent  $a_3'''$ , though so far the experiments have not supported such a possibility.

#### C. *Effect of light on the $a_3''$ CO compound.*

Considering that the CO inhibition of respiration is light sensitive, the effect of light on  $a_3''$  CO is of special interest. In our experiments the spectrum of  $a_3''$  CO is not affected even when a thin and almost transparent layer of our preparation is exposed to a strong source of light. In this respect  $a_3''$  CO behaves very differently from alkaline cytochrome  $c$  or from pyridine haemochromogen, the CO compounds of which, when exposed to light, liberate the CO and revert to the corresponding haemochromogens. In the case of the haemochromogen, however, the CO competes not with oxygen but with pyridine:



The effect of light on  $a_3''$  CO probably becomes apparent only during the catalytic activity of  $a_3$  in presence of oxygen which rapidly oxidizes the reduced  $a_3$  thus preventing its reaction with CO.

#### D. *Respiratory mechanism in absence of cytochrome $a$ .*

Another interesting point to be considered is the respiratory mechanism of some cells devoid of cytochrome  $a$  and yet showing a strong inhibition by KCN as well as a light-insensitive inhibition by CO. In these cells it is conceivable that one of the other components of cytochrome such as  $b$  or  $c$  may have an easily autoxidizable derivative with the properties and functions of  $a_3$  and capable therefore of replacing it. Alternatively, the true cytochrome oxidase may be replaced in these cells, as in cells com-

pletely devoid of cytochrome (spores of *Bacillus subtilis*), by an entirely different enzyme, e.g. a copper-protein compound. This would explain the KCN and light-insensitive CO inhibition of their respiration.

#### IX. OTHER SUPPOSITIONS AS TO THE NATURE OF CYTOCHROME OXIDASE

Before we discuss the results of the experiments described in previous chapters, a few other suggestions as to the nature of cytochrome oxidase may be briefly considered. Thus, the oxidase might be: (1) a compound very similar to components  $a$  and  $a_3$  but present in a concentration too low to be seen spectroscopically, (2) a compound of iron and protein devoid of porphyrin and therefore spectroscopically invisible, (3) an active protein devoid of prosthetic group and functioning as cytochrome oxidase, (4) a compound of copper and protein, or (5) identical with catalase.

The first supposition is inaccessible to experimental test and should be seriously considered only if all other explanations are disproved.

The second supposition, when tested experimentally, gave very inconsistent results. The iron present in the heart-muscle preparation was found to belong mainly to haematin compounds showing different degrees of stability.

The third supposition would imply the existence of an active protein capable of combining with at least one cytochrome component and activating it. On combining with component  $a$ , such a protein may form a complex within which this component reacts with oxygen, carbon monoxide and other respiratory inhibitors forming a series of derivatives spectroscopically recognizable. This supposition is, however, not easily accessible to experimental test.

The two remaining possibilities (4 and 5) should, however, be considered in more detail.

##### A. *Oxidase and intracellular copper-protein*

The supposition that the enzyme may be a copper-protein compound is supported by the following considerations:

- (a) The very wide if not universal distribution of intracellular copper.
- (b) The facility with which copper salts oxidize reduced cytochrome.
- (c) The necessity of copper for development of "indophenol oxidase" and of cytochrome  $a$  demonstrated by culture experiments on yeast and feeding experiments with mice (Elvehjem 1931; Cohen and Elvehjem 1934; Yoshikawa 1937).
- (d) A certain similarity in properties between cytochrome oxidase and

polyphenol oxidase which, as has recently been shown, is a copper-protein compound (Kubowitz 1937; Keilin and Mann 1938).

Owing to its insolubility, attempts to fractionate and purify cytochrome oxidase have met with little success. So far, the highest activity reached for the oxidation of *p*-phenylenediamine in presence of an excess of cytochrome *c* at 38° C corresponds to  $Q_{O_2} = 1400$ . This preparation contained 0.0129 % of non-dialysable copper which can be considered as a fairly high copper content for an insoluble biological material. However, neither the activity nor the copper content have been sufficiently increased to permit the drawing of any definite conclusions as to the relation of the oxidase to a copper-protein compound.

All the above considerations show that the possibility of the copper nature of cytochrome oxidase is based only on indirect evidence. Against this view, moreover, is the fact that the CO compound of copper-protein enzymes is not light sensitive, while the CO poisoning of cells containing cytochrome *a* is sensitive to light.

That copper plays an active part in the formation of cytochrome oxidase has already been mentioned although the mechanism of such activity is not yet understood.

### B. *Oxidase and catalase*

Considering that catalase is always found in cells containing cytochrome and that it is present in the heart-muscle preparations it may be asked whether this enzyme has any connexion with cytochrome oxidase.

At first sight such a supposition seems to be plausible, especially as it is well known that catalase, which is a haematin compound, is affected by and combines with all the inhibitors which affect the oxidase reaction of the cell. This supposition was not, however, confirmed by further study of the problem and is shown to be untenable from the following considerations:

(a) Catalase may be present in a high concentration in cells, e.g. red blood corpuscles of mammals, which are completely devoid of cytochrome oxidase.

(b) Catalase, unlike the oxidase, can be obtained in clear solution.

(c) Unlike the oxidase, it shows great resistance to organic solvents such as alcohol and chloroform which are used for its purification.

(d) Catalase, which is a methaemoglobin-like compound, cannot be reduced by biological systems such as succinic dehydrogenase. It remains in the ferric state even in the presence of strong reducers such as  $\text{Na}_2\text{S}_2\text{O}_4$ .

although it is easily reduced by  $\text{H}_2\text{O}_2$  and reoxidized by molecular oxygen (Keilin and Hartree 1938c).

(e) It does not oxidize reduced cytochrome *c* which is at once oxidized by the muscle preparation.

(f) While  $10^{-4}$  M hydroxylamine inhibits the catalase activity of the muscle preparation by 95 %, it has no effect on the oxidase activity of the same preparation.

All this clearly shows that catalase and cytochrome oxidase are two distinct intracellular catalysts.

## X. DISCUSSION

The study of heart-muscle preparations reveals the existence of a new haematin compound,  $a_3$ ,\* in addition to the components *a*, *b* and *c* of cytochrome. The existence of this compound was not previously recognized because in the oxidized state its absorption bands are invisible while in the reduced state they coincide with the corresponding bands of the component *a*. The evidence for its existence is obtained mainly from the study of the effects of certain respiratory inhibitors on the absorption spectrum of cytochrome. As these substances produce definite modification in the bands of component *a* it appears at first sight that they react directly with *a*. Careful analysis of these reactions reveals, however, that the absorption bands  $a\alpha$  and  $a\gamma$  do not belong to the component *a* only but to a mixture of *a* and  $a_3$ . The effects of the respiratory inhibitors on the appearance of the bands of cytochrome *a* is not due to their reaction with *a* but to the compounds they form with  $a_3$ .

One of the main properties of  $a_3$  is its marked autoxidizability, and in this respect it differs from other components of cytochrome which are considered as non-autoxidizable haematin compounds. The term "non-autoxidizable" should not, however, be taken in too strict a sense because cytochrome *b*, as we have seen, is to a great extent autoxidizable, although the rate of its oxidation by molecular oxygen is not as rapid as that of  $a_3$  or of an ordinary haem or haemochromogen. The components *a* and *c* can also undergo a slow autoxidation even in presence of cyanide. Furthermore, a solution of pure cytochrome *c* cannot be preserved in the reduced state unless it is protected from oxygen. The autoxidation of *a* and *c* is, however, too slow to have any biological significance, while the autoxidation of *b*, although slower than that of  $a_3$ , may play a certain role in biological oxidation reactions.

\* For reference to components  $a_1$  and  $a_2$  see footnote, p. 174.

It may be mentioned here that the property of "non-autoxidizability" is very rare among haematin compounds. It is known only in a few natural compounds such as haemoglobin, heliocorubin ( $> \text{pH } 7$ ) and cytochrome, and so far has never been obtained artificially. In fact, haem and haemochromogen compounds prepared from every type of porphyrin or combined with any nitrogenous substance, as well as the compounds obtained by denaturation of haemoglobin and of cytochrome, are invariably autoxidizable and combine with KCN and with CO.

The mere observation that an intracellular haematin compound is autoxidizable is, therefore, not sufficient to identify it with cytochrome oxidase. It must at the same time react with all the specific inhibitors and be influenced by all the factors which affect the oxidase reaction of the cell. It must also react with at least one of the non-autoxidizable components of cytochrome, of which *c* has already been found essential for the catalytic activity of the oxidase (Keilin and Hartree 1938*a*).

So far, the component  $a_3$  seems to be the only intracellular substance which answers most, although apparently not all, of these requirements.

In fact, the component  $a_3$  is thermolabile and is affected by all treatments such as drying, freezing, acetone, alcohol, acids, alkali, etc., in the same way and to the same degree as is the oxidase activity of the preparation. It is autoxidizable and can be seen to undergo oxidations and reductions during the catalytic oxidation of metabolites. It forms two compounds with KCN: a divalent compound which is easily autoxidizable, and a trivalent compound which does not easily undergo reduction. In the trivalent state it combines with  $\text{H}_2\text{S}$ ,  $\text{NaN}_3$  and  $\text{NH}_2\text{OH}$  which, like KCN, stabilize it and prevent its reduction. In the divalent state it combines with CO forming a compound with bands occupying the same positions (590 and 432  $\text{m}\mu$ ) as the corresponding bands in the photochemical absorption spectrum obtained by Warburg and his co-workers. The component  $a_3$  is therefore the only intracellular haematin compound which may be responsible for this photochemical absorption spectrum.

All this strongly supports the view of the identity of the component  $a_3$  with cytochrome oxidase. This conclusion is, moreover, in agreement with the main results obtained by Warburg who has demonstrated that a haematin compound, which with CO gives an absorption spectrum showing bands at about 590 and 432  $\text{m}\mu$  plays an essential role in cellular respiration. Component  $a_3$  can therefore be identified with Warburg's respiratory or oxygen transporting enzyme.

There are still, however, a few points which require further consideration before the view identifying  $a_3$  and oxidase can be definitely accepted.

Outstanding among them is a direct and convincing demonstration of the reaction between  $c''$  and  $a_3'''$ . Our failure to demonstrate this reaction may be due either to some unrecognized technical difficulties or perhaps to the fact that this reaction is more complicated than it appears and takes place only in presence of molecular oxygen which would reoxidize  $a_3$  as rapidly as it is reduced by  $c''$ . Hence the reduced  $a_3''$  could hardly be expected to be visible spectroscopically.

Two other difficulties (see p. 184) which have not yet received a satisfactory explanation are (1) that strong light has no effect on the spectrum of  $a_3''$  CO, and (2) that there exist cells, the respiration of which is poisoned by KCN and by CO, and yet they are devoid of cytochrome or of its component  $a$ .

In spite of these few points which still remain open for further investigation, the view of the identity of cytochrome oxidase with  $a_3$  seems to be sufficiently well founded to be accepted as the best explanation of all the observed facts discussed in this paper.

As to the relationship between components  $a$  and  $a_3$ , the invariable coexistence and proportionality of these two compounds, the identity of their haem nuclei, a certain similarity in their absorption spectra and in the properties of their proteins suggest that they must be intimately connected if not interconvertible. It is only by a careful study of the effects of various factors on what was always considered as the absorption spectrum of cytochrome  $a$  that the existence of  $a_3$  was revealed. The fact that the main portion of the band lying at  $448m\mu$  belongs to the reduced component  $a_3$  shows that the concentration of this component within the cell is of the same order of magnitude as that of other components of cytochrome.

If we accept now the identity of  $a_3$  with cytochrome oxidase, it is immaterial whether  $a_3$  is considered as an enzyme and  $a$ ,  $b$  and  $c$  as carriers, or whether  $a_3$  is considered as one of the cytochrome components working in a catalytic chain with the other components. In fact, every component of cytochrome can be defined as a catalytically active conjugated protein with a haematin as its active or prosthetic group. Each of these components can, therefore, be considered as an oxidizing enzyme in the same sense as the yellow enzyme.

It must be remembered that all the oxidizing properties of the heart-muscle preparations which can be measured, including the ordinary indophenol reaction, do not belong to one substance, such as  $a_3$ , but are the results of the catalytic activity of the whole cytochrome system.

Cytochrome components form within the cell a highly active catalytic

system which by utilizing molecular oxygen can easily oxidize to water certain hydrogen atoms in the substrate molecules activated by specific dehydrogenase systems.

It is conceivable, however, that in addition to this function some of the cytochrome components may also act as carriers between different dehydrogenase systems, thus playing a part in some anaerobic oxidation-reduction reactions taking place in aerobic cells.

## XI. SUMMARY AND CONCLUSIONS

1. Heart-muscle preparation can be obtained, exhibiting strong cytochrome oxidase and succinic dehydrogenase activities and showing not only the usual  $\alpha^f$  and  $\beta$ -bands of components *a*, *b* and *c*, but also the three Soret or  $\gamma$ -bands lying at 448, 432 and 415  $m\mu$ .

2. In addition to components *a*, *b* and *c* of cytochrome this preparation reveals the existence of a component  $a_3$ , the bands of which are fused with those of *a*. While the main portion of the  $\alpha$ -band (605  $m\mu$ ) belongs to component *a*, most of the  $\gamma$ -band (448  $m\mu$ ) belongs to component  $a_3$ . The bands at 432 and 415  $m\mu$  belong to components *b* and *c* respectively.

3. The existence of  $a_3$  can be demonstrated also in fresh untreated thoracic muscles of insects, in baker's yeast and in strictly aerobic bacteria.

4. Component  $a_3$  undergoes reduction under the same conditions as the other components of cytochrome.

5. Like components *a* and *b*,  $a_3$  is thermolabile and very sensitive to organic solvents, alkali and acids. However, unlike the other components of cytochrome,  $a_3$  is very easily autoxidizable, and in the reduced state combines with CO forming a compound which shows two absorption bands:  $\alpha$ —590  $m\mu$  and  $\gamma$ —432  $m\mu$ .

6. Component  $a_3$  combines with KCN both in the divalent and trivalent states forming two different compounds, of which the former is very easily autoxidizable, while the latter is stabilized in the oxidized state and cannot be easily reduced.

7. In the trivalent state  $a_3$  combines with  $H_2S$ ,  $NaN_3$ ,  $NH_2OH$  and possibly with NaF and  $C_2H_5OOH$ , exhibiting in this respect a close analogy with methaemoglobin, catalase and peroxidase.

8. The main properties of component  $a_3$ , such as its thermolability, autoxidation, reduction by biological systems, formation of reversible compounds with KCN,  $H_2S$ ,  $NaN_3$  and  $NH_2OH$ , which stabilize it in the trivalent state, and with CO, which stabilizes it in the divalent state, strongly support the view identifying this component with cytochrome



oxidase. The catalytic activity of this enzyme depends, however, entirely on co-operation with components *a*, *b* and *c* of cytochrome.

9. Cytochrome oxidase or component  $a_3$  may therefore be identified with the respiratory or oxygen-transporting enzyme of Warburg and his co-workers.

10. There still remain, however, a few weak points in the theory identifying  $a_3$  with cytochrome oxidase. Outstanding among them is the failure to demonstrate a direct or indirect reduction of the oxidized component  $a_3$  by addition of reduced *c* under strictly anaerobic conditions and in complete absence of other reducing substances.

11. The co-existence of *a* and  $a_3$ , their proportionality, the identity of their haem nuclei, the great similarity in some properties of their protein suggest that these two components are very intimately connected if not interconvertible.

12. The concentration of  $a_3$  within the cell is of the same order of magnitude as that of any other component of cytochrome.

13. Component *b* is autoxidizable but not as efficiently as  $a_3$ . It does not combine, however, with CO or other respiratory inhibitors.

14. Narcotics, which in presence of biological reducing systems inhibit the reduction of *a*,  $a_3$  and *c*, inhibit, on the contrary, the oxidation of *b*.

15. Contrary to the statement by other workers it has been demonstrated manometrically that cytochrome *c* does not combine with CO, within the physiological range of pH.

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# Oxygen produced by isolated chloroplasts

BY R. HILL

*From the Biochemical Laboratory, Cambridge*

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## 1. INTRODUCTION

The system concerned with the assimilation of carbon dioxide by the green plant has, under optimum conditions, an activity comparable with the highest rate of cellular respiration in animals. At the moment there is, in the case of animals, considerable knowledge of the subcellular\* chemical mechanisms which can be connected with respiratory activity. In the green plant, on the other hand, there is still no direct indication of a single chemical mechanism connected with carbon assimilation. As the oxygen uptake of tissue preparations apart from  $\text{CO}_2$  evolution is a guide in searching for systems connected with respiration, so might an oxygen output, apart from  $\text{CO}_2$  absorption, indicate mechanisms characteristic of photosynthetic activity in the plant. The subcellular evolution of oxygen under illumination has been known in the case of green plants for many years (Spoehr 1926). The effect, however, was always insignificant compared with the original photosynthetic activity of the cell. The oxygen could only be detected by using certain bacteria which show either motility or luminescence with traces of this gas. But to this method we owe the classical investigations of Engelmann (Spoehr 1926) who showed that in the living cell oxygen appeared in the neighbourhood of the illuminated chloroplast and the experiments on the isolated chloroplasts of *Funaria hygrometrica* by Haberlandt, who demonstrated the production of oxygen in light. Ewart (1896) confirmed and extended these results using other mosses and *Selaginella helvetica*; in a phanerogam, *Elodea*, no oxygen could be observed to come from the isolated chloroplasts.

The same problem was approached in a somewhat different manner by Molisch (1925). The leaves of many phanerogams were allowed to dry

\* The term *subcellular* is used here to imply a degree of organization less than that of the whole cell.

slowly in air and finally over a dehydrating agent. This produced a stable preparation which, if ground up in water, would show an evolution of oxygen in light which could be detected by the bacterial methods. Molisch showed that these preparations were thermolabile, indicating an enzymic process. Recently, the matter was taken up by Inman (1935) who confirmed the experiments of Molisch and showed also that fresh green extracts of many phanerogams will evolve oxygen in light, using the bacterial method. Inman brought further evidence as to the enzymic nature of the process, and, moreover, did not consider the oxygen evolved to represent photosynthesis but suggested that it was due to a limited store of oxygen-giving material.

This subcellular evolution of oxygen is then the only property specific for green plant tissue which is at the moment open to biochemical investigation. So far the oxygen had been detected qualitatively by two methods, both using bacteria. Two questions then naturally arise: can molecular oxygen be proved by an independent method, and can the activity of preparations from green tissue be measured and compared with the activity of the living cell?

It happens that there is one reagent which can be used to detect and measure traces of oxygen with certainty in a liquid medium. This reagent is haemoglobin, and it has this rare property of combining with molecular oxygen without being oxidized by it. The absorption spectra of oxyhaemoglobin and haemoglobin are very different and oxyhaemoglobin possesses so strong an absorption of light that it can be used in a dilution corresponding to concentrations of oxygen from  $10^{-5}$  to  $10^{-4}$  M. But as oxyhaemoglobin is a dissociable compound, the method is limited by the affinity of the haemoglobin for oxygen. However, it has this advantage, that not only can the amount of oxygen evolved be determined but also the pressure of oxygen obtaining in the fluid. Hoppe-Seyler (1879) demonstrated the use of mammalian blood in detecting the oxygen produced by the green plant in carbon assimilation. Observations on the affinity of muscle haemoglobin (Hill 1933; Theorell 1934) for oxygen suggested that this substance could be used as a sensitive method both for detecting and measuring the oxygen given off by isolated chloroplasts (Hill 1937). This spectroscopic method is much less sensitive than the bacterial methods. However, it was shown (Hill 1937) that suspensions of chloroplasts of many angiosperms can under suitable conditions give measurable amounts of oxygen in light.

The fresh suspensions of chloroplasts obtained by crushing leaves in sucrose solution would not, on illumination, evolve measurable amounts

of oxygen even in the presence of  $\text{CO}_2$ . But in the presence of an aqueous extract of acetone-treated leaf, oxygen was evolved in the light. These two findings substantiate the observations of the earlier workers and at the same time justify the contentions of Kny (1897) that the chloroplast was not perhaps a complete photosynthetic system in itself.

The present paper is devoted to an examination of this oxygen-producing property of chloroplasts. The results obtained show that for the purpose of biochemical investigation the activity of chloroplasts removed from the cell is significant, being about one-tenth the activity of the living leaf.

*General procedure.* The fresh leaves of a plant are crushed with a pestle and mortar in sucrose solution. The resulting mass is strained through glass-wool. The suspension of chloroplasts is then introduced into an evacuated Thunberg tube containing haemoglobin and desired reagents. The tube is then illuminated and oxygen estimated spectroscopically as oxyhaemoglobin.

## 2. PREPARATION OF SUSPENSIONS OF CHLOROPLASTS AND OF MESOPHYLL CELLS

The two species of flowering plant used in the present work were *Stellaria media* and *Lamium album*. Similar results however have been obtained with a variety of angiosperms. In the case of *Stellaria media* the leafy stems were picked between 9 and 10 a.m., the leaves detached and soaked in tap water for 45 min. in a diffuse light. The chloroplasts were extracted by grinding 0.5 g. of the leaves for 1 min. with 2.5 c.c. of 10% sucrose in M/30 phosphate pH 7.9. The mass was then poured into a plug of glass-wool in a funnel; the crushed tissue was almost completely retained while the green suspension containing most of the chloroplasts was collected. The suspension, 0.2–0.5 c.c., was then at once introduced into an evacuated Thunberg tube containing 5 c.c. of a solution of the haemoglobin and other substances under investigation. All these manipulations were performed in diffuse daylight. The small quantity of oxygen introduced could either be removed by evacuation or allowed to remain in the tube and measured at the beginning of an experiment. The tube was then exposed to a strong light and any oxygen measured as oxyhaemoglobin with the spectro-colorimeter.

In the case of *Lamium album*, the leaves, treated as previously described, were ground with 10% sucrose containing 0.5% of sodium potassium tartrate (Rochelle salt). The fluid was strained through glass-wool and

centrifuged at 1000 r.p.m. for 6 min. The unbroken cells and some of the chloroplasts then remained packed at the bottom of the centrifuge tube. The fluid was poured off and centrifuged for 5 min. at 1800–2000 r.p.m. This gave a deposit of chloroplasts only. The fluid, which was still green, was discarded and the sediment gently stirred into a suspension with a little fresh sucrose solution. It was also possible to remove the unbroken cells completely by filtering the original fluid through a wad of absorbent cotton-wool pressed tight to 1 cm. thickness while wet. In this case also it was necessary to centrifuge out the plastids and resuspend them in fresh fluid; the aqueous extract of *Lamium album* contains tannins which interfere with subsequent operations.

The chlorophyll content of a suspension of chloroplasts or mesophyll cells was estimated by adding 4 vol. of acetone and extracting the fluid with toluene. The toluene solution was then determined by the Nutting spectrophotometer. The value of  $\log_{10} I_0/I$  at 6750 Å was taken to be 4.8 for a  $10^{-4}$  M solution of chlorophyll in toluene ( $1b + 3a$ ) 1 cm. in thickness. The vacuum tubes used were 1.5 cm. internal diameter and the final concentration of chloroplasts, represented as a concentration of chlorophyll was between 0.2 and  $0.5 \times 10^{-4}$  g.mol./l.

### 3. THE MEASUREMENT OF OXYGEN EVOLVED

The chloroplasts were suspended in a liquid medium containing a known quantity of haemoglobin in a vacuum tube. The relative concentrations of haemoglobin and oxyhaemoglobin were determined in a spectro-colorimeter which has been described elsewhere (Hill 1936). This method of measurement was used previously for spectroscopic determination of oxygen dissociation curves. The presence of  $0.2\text{--}0.5 \times 10^{-4}$  M chlorophyll, while showing a strong band in the red, does not interfere with the part of the spectrum used. If a glass cell containing a suspension of chloroplasts of suitable concentration is placed in the light beam illuminating the standard, two perfectly similar spectra can be obtained. This refinement was found, however, not sensibly to increase the accuracy.

The muscle haemoglobin was obtained from shin beef by a method based on that of Theorell (1932). It was important to have a preparation which did not change readily to methaemoglobin, so the purification was not carried further than the addition of basic lead acetate, removal of the lead from the filtered fluid, and dialysis.

The method was as follows: 4 lb. of shin beef after freeing from fat were cut up and rolled in about 100 g. precipitated  $\text{CaCO}_3$  and minced. This gave an intimate

mixture with the  $\text{CaCO}_3$ . About 700 c.c. of distilled water were added and after 10 min., stirring at intervals, the mass was rapidly squeezed in portions through a cloth. The fluid was mixed with about 30 g. Kieselguhr and rapidly filtered through a layer of Kieselguhr on a large Buchner funnel. The filtrate, 700 c.c., was treated at once with 92 c.c. basic lead acetate (B. P. Fort). After removal of the precipitate by filtration the excess Pb was removed by disodium phosphate added rapidly to reach a pH of 7.5–8. After centrifuging, the clear fluid was dialysed against either tap or distilled water for 24–36 hr. This preparation has the property of keeping free from methaemoglobin for several days when kept at 0° C. If, however, the first process is carried out too slowly or not enough lead acetate added, the preparation rapidly formed methaemoglobin.

The oxyhaemoglobin solution usually contained about  $1.8 \times 10^{-4}$  g. atom of haemoglobin iron per litre. The strength of this stock solution was measured in the spectrophotometer. The value of  $\log_{10} I_0/I$  at 5810 Å was taken to be 1.6 for a solution containing  $10^{-4}$  g. atom of haemoglobin iron per litre.

Supposing at the beginning of the experiment we have, in the vacuum tube, a suspension of chloroplasts in a fluid containing a known quantity of haemoglobin. The oxygen has been almost completely removed and the haemoglobin will be less than 5% saturated with oxygen. If, then, minute amounts of oxygen are added to the tube (e.g. 0.5 c.c. water saturated with air), the spectrum of oxyhaemoglobin will appear, and the increase in intensity of the absorption bands is a measure of the oxygen added to the liquid in the tube.

The loss of oxygen from the liquid to the vapour phase in the vacuum tube was found to be negligible under the conditions of the experiments. If, however, the tube is shaken thoroughly and continuously, 3 min. are required to attain equilibrium with the vapour phase. Thus it is easy to remove most of the oxygen from the liquid during an experiment without opening the tap to a vacuum.

If no oxygen is added from outside, an increase in saturation of the haemoglobin is a measure of the oxygen liberated from some source in the fluid. The affinity of muscle haemoglobin for oxygen is so high that the concentration of free oxygen in the solution is negligible under the conditions used. The results of illuminating chloroplasts under different experimental conditions are given graphically as the percentage saturation of the haemoglobin with oxygen in relation to the time of illumination. The concentration and variety of the haemoglobin is stated, also the temperature and pH. From this not only can the quantity of oxygen liberated be calculated but also the pressure of oxygen in apparent equilibrium with the system at any moment. Both these quantities it is of importance to measure.

In fig. 1 are shown curves giving the percentage saturation of the haemoglobin at different pressures of oxygen. A solution of haemoglobin containing  $0.45 \times 10^{-4}$  g.atoms of haemoglobin Fe per litre represents 1 c.mm. of oxygen per c.c. when fully saturated. The pressure of oxygen for half saturation of muscle haemoglobin at  $20^{\circ}\text{C}$ , pH 7.9 is 0.6 mm. Hg. For dilute human haemoglobin from blood under the same conditions, the corresponding oxygen pressure is 1.7 mm. Hg. It can be seen from the graph that the muscle haemoglobin is suitable for detecting low pressures of oxygen.

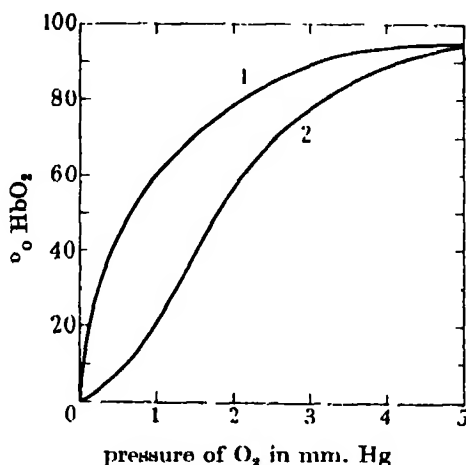


FIG. 1. Oxygen dissociation curves of haemoglobins at  $19^{\circ}\text{C}$  and pH 8. Curve 1, muscle haemoglobin; curve 2, haemoglobin from human blood.

In order to obtain the evolution of measurable amounts of oxygen a strong source of light was used. A concentrated beam from a projection lens was used and the illumination was approximately equal to that obtainable from a 1200 c.p. lamp 6 in. from the vessel. This light source was kept constant for all the experiments described in the present paper.

#### 4. PREPARATION OF EXTRACTS FROM LEAVES

The chloroplasts of *Lamium album* or *Stellaria media*, when suspended in solutions of sucrose, would not evolve oxygen in the light even in the presence of  $\text{CO}_2$ . If, however, they were suspended in a fluid prepared from an acetone powder of the leaves, oxygen was evolved in the light and rapidly taken up in the dark. The extracts were made as follows:

40 g. fresh leaves of *Lamium album* were ground in a mortar with 160 c.c. of acetone until the chlorophyll was dissolved. The powder was

filtered off and washed first with 80% acetone and finally with pure acetone. Then the powder was allowed to dry. 2 g. of the powder were stirred up with 20 c.c. of water and the fluid filtered off at once by suction through Kieselguhr. This extract was best kept *in vacuo* to avoid browning due to oxidation. The addition of a fresh extract of the leaf prepared in this way did not form methaemoglobin when mixed with muscle haemoglobin. In these extracts from leaves, the property of producing oxygen with chloroplasts was thermolabile. In many cases only traces of iron were initially present and ferric salts (as shown by the reaction with  $\alpha\alpha'$ -dipyridyl and a reducer) could be removed completely without loss of activity.

It was found that an extract of acetone yeast prepared in a similar way would yield oxygen in the presence of chloroplasts. Also an extract made by boiling yeast in water for a short time. In an attempt to fractionate the active part of the yeast extract, it appeared to be ferric iron compounds of organic acids. Moreover, after a preliminary adsorption on tricalcium phosphate, and liberation by oxalate, the oxygen output was nearly proportional to the ferric iron content as estimated by  $\alpha\alpha'$ -dipyridyl. Then it was found that ferric potassium oxalate when added to a suspension of chloroplasts caused the evolution of oxygen in a quite startling manner on illumination. In this case the oxygen uptake due to the ferrous oxalate formed was less rapid so that a much higher partial pressure of oxygen could be reached than with leaf extracts.

#### 5. CHLOROPLASTS AND FERRIC SALTS

When ferric potassium oxalate was added to muscle oxyhaemoglobin at pH 8, no reaction took place. If now the mixture was evacuated, as the oxygen was removed, some methaemoglobin was formed along with the reduced haemoglobin. If all the oxygen was removed and the fluid exposed to white light no further change took place.

On the other hand, if a large excess of ferric potassium oxalate was added to methaemoglobin in the presence of oxygen and the mixture exposed to white light, the spectrum of oxyhaemoglobin gradually appeared. As the ferric iron became reduced photochemically at the expense of some of the organic substances, the ferrous iron formed would then reduce the methaemoglobin to haemoglobin which then became oxygenated.

The presence of oxygen therefore shifts the equilibrium of the iron oxalate-haemoglobin system towards more reduction of the haemoglobin, owing to the oxygenation of the latter. This was shown clearly by adding a



trace of ferrous potassium oxalate to methaemoglobin in the presence of air; oxyhaemoglobin was produced at once. If, however, the experiment was performed *in vacuo*, very little reduction to haemoglobin occurred. It also follows that ferrous potassium oxalate is oxidized more quickly by methaemoglobin than by oxygen. Hence the formation of methaemoglobin when ferric oxalate is present *in vacuo* does not interfere with measurement of molecular oxygen subsequently present. With haemoglobin of blood, however, there is no formation of methaemoglobin.

If a mixture of ferric potassium oxalate and muscle haemoglobin was completely deprived of oxygen in the presence of chloroplasts, exposure to light rapidly produced oxyhaemoglobin. In the dark the oxygen was slowly absorbed, the system tending to approach the state before illumination. If  $\alpha\alpha'$ -dipyridyl was added after illumination, the red colour was immediately produced indicating reduction of the iron to the ferrous state.

The percentage of the saturation of the haemoglobin was measured and plotted against time.

The fluid in the vacuum tube contained: 1 c.c. of M/2 potassium oxalate, 0.7 c.c. of water containing the necessary amount of ferric potassium oxalate, 1 c.c. of M/15 phosphate buffer pH 7.9 and 2 c.c. of a stock solution of oxyhaemoglobin. The oxygen was first removed completely and then 0.3 c.c. of a chloroplast suspension added through the side tube.

Oxygen evolutions with different amounts of iron are shown in fig. 2*a*. In Table I some figures are given showing the total amounts of oxygen evolved under various conditions. The evolution of oxygen is seen to be proportional to the ferric iron added and corresponds almost to 1 mol. of oxygen for 4 ferric iron atoms. If the amount of chloroplasts was varied the total evolution is not greatly affected, but the rate was altered (fig. 2*b* and Table II). The amount of ferric iron added does not greatly alter the initial rate above  $0.5 \times 10^{-4}$ . Below this the rate fell off (Table III). The initial rate with ferric oxalate is very nearly the same as with the chloroplasts in presence of leaf extracts which contain no oxalate and very little ferric iron; this is shown in fig. 3. In Table II the initial rate of oxygen production is expressed in terms of molecules of oxygen produced per hour per molecule of chlorophyll present. This again is converted by the factor 44/900 to the equivalent value of the photosynthetic number of Willstätter, represented here as *Pc* (Spoehr 1926).

The effect of intensity of illumination was not studied in detail because the experimental conditions were not suitable. The thickness of the vessel being 1.5 cm., a concentration greater than  $0.5 \times 10^{-4}$  M chlorophyll could

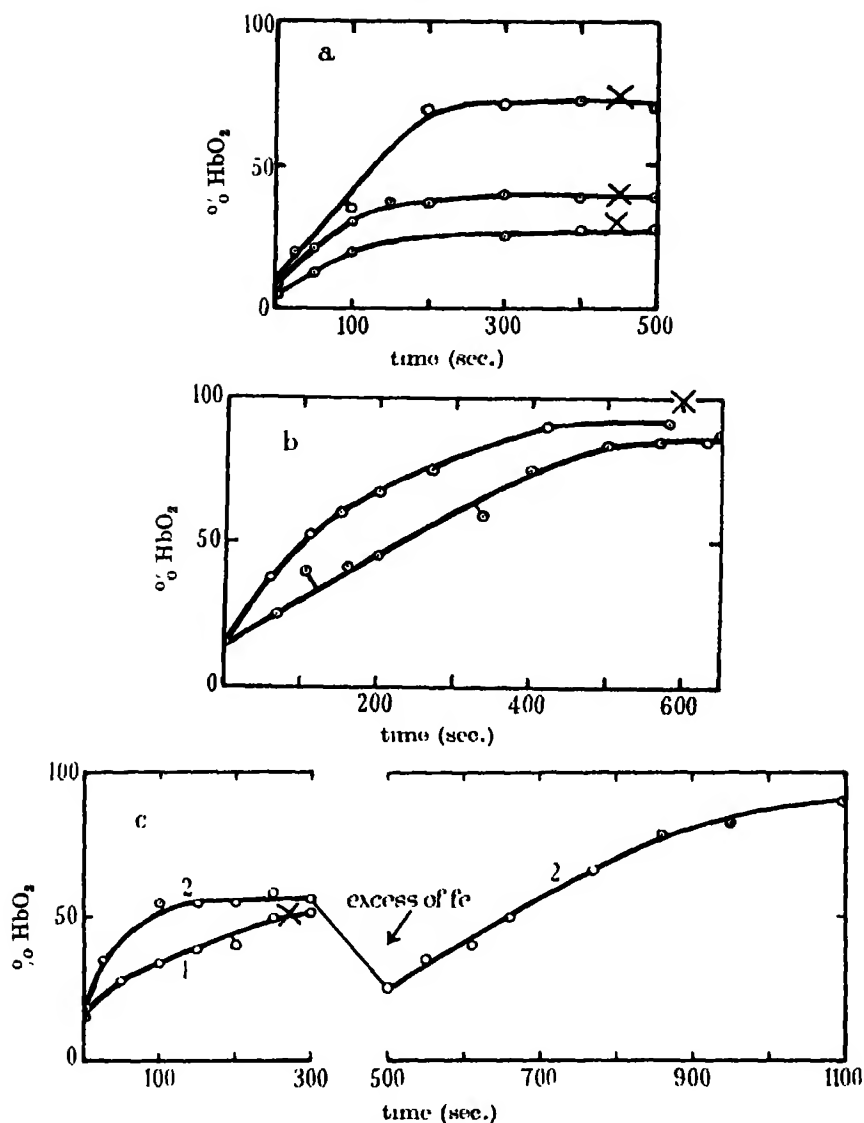


FIG. 2. Chloroplasts of *Stellaria media* in ferric oxalate and muscle haemoglobin. 20° C, pH 8.

The crosses represent the theoretical saturation of the haemoglobin for equation (2) in text. (a) Three curves corresponding to ferric iron concentrations of  $0.76 \times 10^{-4}$ ,  $1.0 \times 10^{-4}$  and  $2.0 \times 10^{-4}$  g.atoms/l.; haemoglobin  $0.6 \times 10^{-4}$ . (b) Two curves corresponding respectively to two chloroplast concentrations  $0.17 \times 10^{-4}$  and  $0.35 \times 10^{-4}$  M chlorophyll; ferric iron  $4 \times 10^{-4}$ , haemoglobin  $1 \times 10^{-4}$ . (c) Two curves corresponding respectively to two chloroplast concentrations  $0.15 \times 10^{-4}$ ,  $0.5 \times 10^{-4}$  M chlorophyll; ferric iron  $1 \times 10^{-4}$ , haemoglobin  $0.7 \times 10^{-4}$ . Curve 2 shows the effect of removing oxygen by evacuation and then adding an excess of ferric oxalate.

TABLE I. EFFECT OF FERRIC OXALATE AND CHLOROPLAST CONCENTRATION ON TOTAL OXYGEN PRODUCED

Exp.	Concentration in g.atoms of iron per litre		Chloroplasts corresponding to molecular concentration of chlorophyll	Total increase in % saturation of the haemoglobin during exposure to light	Equivalents O Equivalents Fe
	Muscle haemoglobin	Ferric oxalate			
p. 257	$1.0 \times 10^{-4}$	$2.0 \times 10^{-4}$	$0.2 \times 10^{-4} \dagger$	46	0.92
	$1.0 \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.2 \times 10^{-4} \dagger$	75	0.75
p. 255	$0.6 \times 10^{-4}$	$0.76 \times 10^{-4}$	$0.3 \times 10^{-4}$	22	0.69
	$0.6 \times 10^{-4}$	$1.0 \times 10^{-4}$	$0.3 \times 10^{-4}$	32	0.76
	$0.6 \times 10^{-4}$	$2.0 \times 10^{-4}$	$0.3 \times 10^{-4}$	65	0.78
p. 256	$1.0 \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.17 \times 10^{-4}$	75	0.75
	$1.0 \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.35 \times 10^{-4}$	76	0.76
p. 276	$0.7 \times 10^{-4}$	$1.0 \times 10^{-4}$	$0.3 \times 10^{-4}$	45	1.2
	$0.7 \times 10^{-4}$	$2.0 \times 10^{-4}$	$0.3 \times 10^{-4}$	80	1.1
p. 271	$1.0^* \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.3 \times 10^{-4}$	82	0.82

\* Blood haemoglobin.

† *L. album*.

TABLE II. EFFECT OF CHLOROPLAST CONCENTRATION ON INITIAL RATE OF OXYGEN EVOLUTION

Exp.	Concentration in g.atoms of iron per litre		Concentration of chloroplasts as chlorophyll in g.mol. per litre	Time in sec. for saturation of Hb; initial rate	Mol. O <sub>2</sub> per hr. Mol. chlorophyll P.c.	
	Hb	Ferric oxalate				
p. 256	$1.0 \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.17 \times 10^{-4}$	610	35	1.5
	$1.0 \times 10^{-4}$	$4.0 \times 10^{-4}$	$0.35 \times 10^{-4}$	290	35	1.5
p. 278	$0.7 \times 10^{-4}$	$1.0 \times 10^{-4}$	$0.15 \times 10^{-4}$	430	40	1.8
	$0.7 \times 10^{-4}$	$1.0 \times 10^{-4}$	$0.5 \times 10^{-4}$	310	16	0.7

TABLE III. EFFECT OF FERRIC OXALATE CONCENTRATION ON INITIAL RATE OF O<sub>2</sub> EVOLUTION WITH CHLOROPLASTS OF *S. MEDIA*

Exp.	Concentration of Fe in g.atoms per litre		Initial rate. Time in sec. for saturation of Hb
	Hb	Ferric oxalate	
p. 276	$0.7 \times 10^{-4}$	$1.0 \times 10^{-4}$	290
	$0.7 \times 10^{-4}$	$2.0 \times 10^{-4}$	200
p. 275	$0.7 \times 10^{-4}$	$0.5 \times 10^{-4}$	740
	$0.7 \times 10^{-4}$	$2.0 \times 10^{-4}$	250
	$0.7 \times 10^{-4}$	$6.0 \times 10^{-4}$	320

not be used. At the lower concentration,  $0.3 \times 10^{-4}$ , it was found that cutting off all wave-lengths shorter than 6000 Å with red glass filter, produced no appreciable effect on the reaction. Under the conditions of these experiments the reaction goes as fast in red as in white light. This shows that chlorophyll is acting as part of the light-sensitive system and not the ferric oxalate.

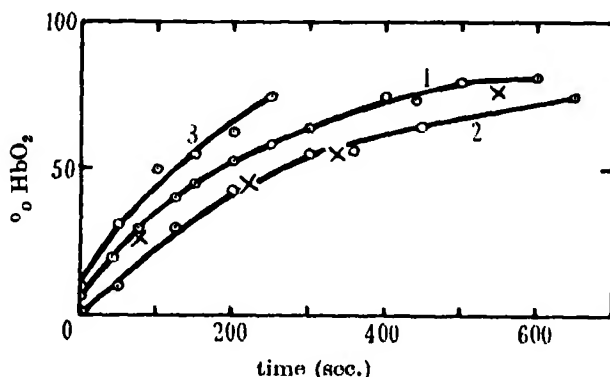


FIG. 3. Chloroplasts in leaf extracts and muscle haemoglobin. (1) *Stellaria media* plastids in extract from *Lamium album* Hb,  $0.9 \times 10^{-4}$ . (2) *Lamium album* plastids in extract from *Anthriscus sylvestris* Hb,  $0.9 \times 10^{-4}$ . The crosses represent the same plastids in ferric oxalate  $4 \times 10^{-4}$ . (3) *Stellaria media* plastids in extract from *Alliaria officinalis* Hb,  $0.44 \times 10^{-4}$ .

The production of oxygen from  $\text{CO}_2$  in the living green plant is inhibited by moderate concentrations of cyanide and hydroxylamine. It had been suggested that catalase (present in almost all plant cells) was concerned in the liberation of oxygen, although more recently indirect evidence against catalase has accumulated (Emerson 1936). Hence the effect of substances like azide, cyanide and hydroxylamine must be determined. Here, however, was a difficulty; the ferric oxalate, as has been mentioned earlier, reacts with muscle haemoglobin to form methaemoglobin. The methaemoglobin combines with azide, etc., to give compounds which are not easily reduced; thus the whole method of detecting and measuring oxygen would break down. It was found, however, that fresh diluted human blood about 1/50, could be mixed with dilute hydrocyanic acid and ferric oxalate at pH 8 in a vacuum without any immediate reaction taking place. While the affinity for oxygen of human blood haemoglobin is much less than that of muscle haemoglobin, the rate of reoxidation of the ferrous oxalate did not make it impossible to measure the oxygen evolved in light. Fig. 4 shows the effect of cyanide and azide and hydroxyl-

amine on the evolution of oxygen. In the case of hydroxylamine a certain amount of combination with methaemoglobin occurred—if this is allowed for, the oxygen output does not appear to be influenced. The same effect occurred with cyanide, but the experiments show a relatively small oxygen deficiency with two widely differing cyanide concentrations.

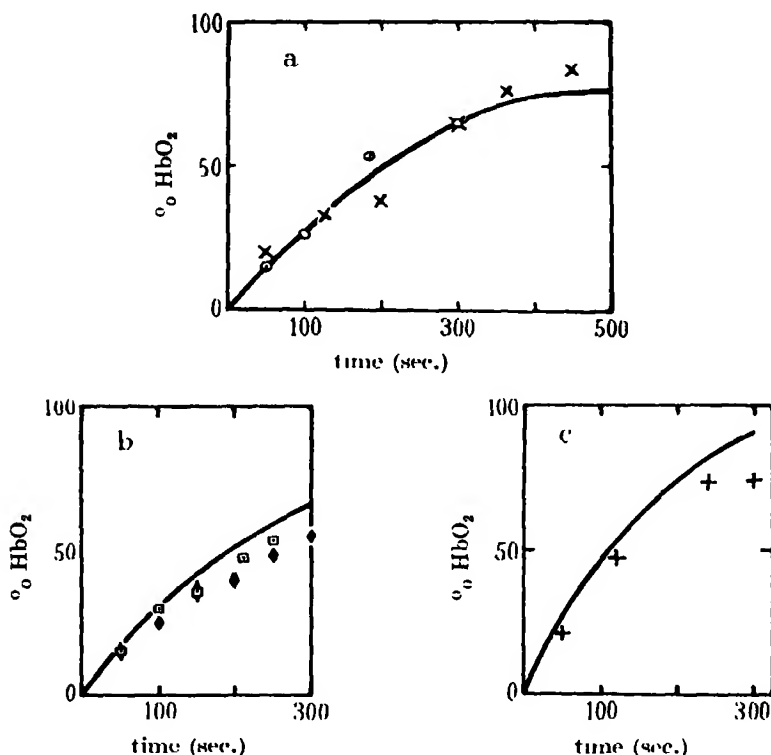


FIG. 4. Effect of certain inhibitors. Chloroplasts of *Stellaria media* in ferric oxalate,  $4 \times 10^{-4}$ , and haemoglobin from human blood,  $1 \times 10^{-4}$ . 20° C, pH 8. a. Curve, no inhibitor. Crosses, sodium azide,  $4 \times 10^{-4}$  M. Circles, sodium fluoride,  $4 \times 10^{-4}$  M. b. Curve, no inhibitor. Squares, HCN,  $3 \times 10^{-4}$  M. Diamonds, HCN,  $60 \times 10^{-4}$  M. c. Curve, no inhibitor. Crosses, hydroxylamine,  $0.2 \times 10^{-4}$  M.

Thus the oxygen output does not seem to be influenced by substances which have an effect on many oxidations and which also combine with catalase and with methaemoglobin. The combination with the last mentioned we use as evidence, that when the oxygen output is measured from illuminated chloroplasts, the effect is not due to some property of the haemoglobin.

That hydrogen peroxide is not directly involved in the reaction producing molecular oxygen it seems safe to conclude from the above facts. An additional point of evidence is that a relatively high concentration (about  $10^{-5}$  M) of peroxidase with the chloroplasts was without influence on the evolution of oxygen. (I am grateful to Professor Keilin and Dr Mann for a preparation of horseradish peroxidase.)

However, it is possible that other peroxides may be involved. This is made unlikely by the fact that we could have a moderate concentration (M/20) of thioglycollic acid present with chloroplasts and yet have no influence on the production of oxygen.

When a mixture of ferric oxalate, chloroplasts, haemoglobin and thioglycollic acid was illuminated, the original deep purple colour of the ferric—SH complex is gradually bleached as the oxyhaemoglobin appeared, showing the reduction of the iron, simultaneously with the appearance of oxygen; at the end of the experiment the strong SH reaction was still obtained.

These facts make it seem improbable that, in this formation of molecular oxygen by the chloroplasts, peroxides play any significant part—at least we cannot demonstrate any peroxide stage, at present.

When cytochrome *c* was added it was almost completely reduced while the oxygen was being evolved in presence of ferric oxalate, and there was no effect on the oxygen output. On the other hand, free haematin greatly diminished the output of oxygen, because it is autoxidizable when reduced.

Only a limited number of ferric compounds could be used as a source of photolytic oxygen in the experiments. Citrate and tartrate could be used instead of oxalate, but the evolution of oxygen was much less rapid in these two cases, and it was necessary to add a large excess of ferric iron. Ferric pyrophosphate or malate showed no reaction with chloroplasts. In many other cases, however, the iron was not in true solution as a complex ion and in these cases there was no reaction with the chloroplasts. Thus it seems probable that the reagent, such as ferric oxalate, used for producing an oxygen output, must have free access to the surface of the chloroplasts. This assumption is supported by the fact that a great dilution of the fluid used when the chloroplasts are removed from the cells produces no influence on the rate of reaction with ferric oxalate. If, however, ferric potassium oxalate is used in the absence of excess of oxalate no reaction occurs with chloroplasts. There must always be an excess of the oxalate ion as compared with the iron present.

A perfectly fresh suspension of chloroplasts which is free from other matter has a characteristic silky appearance when agitated, similar to a

suspension of red blood corpuscles. At this stage the chloroplasts appear on examination to be similar to those in the cells. On keeping the suspension at 20 or at 0° C in the dark the appearance of the suspension soon changes, it becomes darker in colour and less silky on agitation. At the same time the chloroplasts are seen to be losing their clear regular outlines, although they do not appear to be actually breaking up. Before this visible alteration is reached the suspension will be found to be incapable of producing O<sub>2</sub> in the light under any of the experimental conditions. But it must be emphasized that the property of evolving oxygen in light is not proved to be due only to uninjured chloroplasts for the following reasons. After drying the leaf (following Molisch) a slight oxygen evolution up to 0.6 mm. pressure of O<sub>2</sub> is detectable by the haemoglobin method. Owing to the large amount of opaque material necessary for this experiment no actual measurements are given at present. Again, after crushing a living leaf in distilled water, when most of the plastids are broken, this fluid, when the cells are removed, will behave for a short time (2 min.) like a suspension of intact chloroplasts.

#### 6. EVOLUTION OF OXYGEN BY MESOPHYLL CELLS OF *LAMIMUM ALBUM*

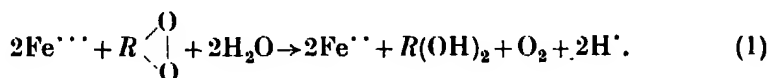
The cell suspension obtained from the leaf by centrifuging (see § 1) contained also free chloroplasts. It was introduced into a tube containing haemoglobin and all the oxygen removed by evacuation. In order to produce a measurable output of oxygen it was necessary to add some CO<sub>2</sub>. 0.2 c.c. of a saturated solution of CO<sub>2</sub> in air-free water was added to the tube through the side arm. The rate of O<sub>2</sub> production per unit of chlorophyll obtained by these preparations was only half that obtained from the chloroplasts in ferric oxalate under similar conditions. Allowing for the presence of inactive chloroplasts outside the cells, the active chloroplasts inside the cells did not greatly exceed the rate of O<sub>2</sub> production of the isolated chloroplasts in the ferric oxalate. Moreover, the CO<sub>2</sub> remaining in the fluid after evacuation is shown to be very small in amount. Hence the oxygen evolved by the isolated chloroplasts in ferric oxalate must all come from the change from ferric to ferrous iron and not from CO<sub>2</sub>—as indeed is shown by the relation of the original ferric iron concentration and the oxygen evolved. To remove with certainty all the CO<sub>2</sub> from a suspension of chloroplasts before they have become inactive is at present impossible, but this is at the moment an essential step in elucidating the mechanism of the formation of oxygen. The probability is, however, that CO<sub>2</sub> does not take part in the production of oxygen from ferric salts as no

difference was found after attempting to remove all the  $\text{CO}_2$  at pH 6.8 by evacuation, and after the addition of  $\text{CO}_2$ .

### DISCUSSION

The first question to decide is whether the isolated chloroplasts are acting in a catalytic capacity or whether they possess some store of oxygen which is only liberated in light. Inman (1935) considered that the oxygen he had detected came from a store of oxygen-producing substance. Kautsky (1938) points out that ferric oxalate causes decomposition of peroxides in light. This latter effect would be far the simplest qualitative explanation of the experiments where ferric oxalate is concerned.

The reaction would be of the type



In the present experiments only half the oxygen for this reaction is obtained. It is possible that some oxygen is lost by other oxidations; yet this is improbable because the oxygen/iron ratio is constant for a series of experiments with different initial concentrations of ferric iron.

The second inference from the experiments is that if there is a store of a peroxide it must be at least  $1/10$  M in the chloroplasts, because at least 5 mol. of  $\text{O}_2$  can be liberated per molecule of chlorophyll. This large quantity of a peroxide would be of great interest if it could be detected by a direct method, but so far there is no evidence for it.

The third inference from the experiments is that there is something which can be extracted from leaves which contains neither  $\text{Fe}^{+++}$  nor oxalate; this however behaves towards chloroplasts very much like ferric oxalate. We will therefore leave aside the question of the store of oxygen-giving substance and consider the chloroplast as a catalytic system.

The conclusion, then, to be drawn from the present investigation is that light energy can be utilized by a subcellular system containing chlorophyll; the work done can be measured in terms of the production of molecular oxygen and reduction of a ferric complex salt.

With potassium ferric oxalate the photochemical reaction in presence of chloroplasts gives nearly the theoretical yield of molecular oxygen for the reaction

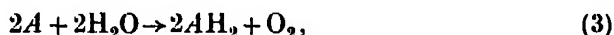


In the dark the reaction will proceed in the reverse direction.



The oxidation-reduction potential of the iron oxalate system is  $E_0H =$  zero at pH 8 (Michaelis and Freidheim 1931), while the potential of the oxygen electrode at a pressure of 1 mm. of oxygen at pH 8 would be  $EH = 0.7$  V. The reduction of the iron goes nearly to completion, but even if 99 % of the iron were reduced the change in free energy would be less than half that required for a direct reduction of carbon dioxide. However, the maximum energy has not been measured as no definite equilibrium could be shown with ferrous and ferric iron in the system.

A suspension of chloroplasts will also evolve oxygen under illumination when in presence of other ferric complex salts with organic acids, and also from extracts of leaves that contain calcium ions and do not give reactions for iron salts. Thus the chloroplasts are not specific for ferric oxalate. The ferric oxalate could then be regarded simply as a reagent to demonstrate a property of the chloroplast. This might be compared to the use of indophenol reagent by Keilin (1929) in measuring the activity of the oxidase cytochrome system in the respiration of cells. There must therefore be some primary substance which is reduced, while at the same time giving oxygen. If this primary substance is  $A$ ,\* and the reagent  $B$ , such as ferric oxalate, represented in terms of hydrogen transport, we have the following reactions.



These two reactions together will represent the type of reaction (2). It must be concluded that the substance  $A$  is not easily removed from the chloroplasts because great dilution of the suspending fluid did not diminish the rate of reaction with ferric oxalate.

With chloroplasts alone we obtain no oxygen either because  $A$  is present in small amount or that a catalyst is needed to oxidize  $AH_2$ . But if a reagent is added which will reoxidize  $AH_2$  sufficiently fast,  $O_2$  can accumulate, but this is only possible if the reduction of the reagent  $B$  is more rapid than the oxidation by molecular oxygen. Thus the conditions are limited in which it is possible to produce a measurable oxygen output with the preparations of isolated chloroplasts.

In the absence of a reagent  $B$ , no oxygen evolution could be detected, that is, it would be less than 1/10 mm. of mercury pressure.  $CO_2$  was also found to exert no influence on the evolution of oxygen in the case of substances which could be reduced. The next most important step would be to ascertain whether  $CO_2$  is actually the primary substance  $A$ , giving

\* This is not necessarily the substance  $A$  in Kautsky's (1937) scheme.

oxygen in the experiments. This cannot be decided directly from the results of the present work. If, however,  $\text{CO}_2$  is the primary substance concerned with oxygen output by isolated chloroplasts, it follows directly from the experiments that the first product of reduction of the  $\text{CO}_2$  must be as rapidly oxidized as it is formed, and hence it could not be normal carbohydrate. Also it must react with ferric oxalate at great speed. Again, if  $\text{CO}_2$  is the primary substance undergoing reduction, the chloroplasts must have the greater part of the photosynthetic mechanism intact. This last assumption is rendered unlikely by the behaviour with cyanide and other poisons. Thus while it is not possible to show directly that  $\text{CO}_2$  plays a part in this system giving oxygen, there is circumstantial evidence against this assumption.

It seems then, that the isolated chloroplasts have very definite and peculiar catalytic properties; it is therefore of use to bring the conclusions into relation with the living cell. This is done, not in order to add yet another theory of carbon assimilation to the many existing, but in order to prepare ground for future experiments, concerned with the evolution of oxygen.

The activity of the isolated chloroplasts measured as rate of oxygen output per unit of chlorophyll is only about 1/10 the activity of the leaf under optimum conditions. When, however, the oxygen production from  $\text{CO}_2$  of isolated mesophyll cells of *Lamium album* is compared with the oxygen production from ferric oxalate or leaf extract with isolated chloroplasts of the same plant, the activity is nearly the same. We may therefore be justified in assuming that as molecular oxygen is a photochemical product in each case, so is the isolated chloroplast behaving in part as it does in the intact cell. In crushed leaf tissue when all cells are broken it can be inferred that oxygen is actually evolved by chloroplasts in light but practically as readily absorbed again. For it is a matter of common experience that crushed green tissue will not assimilate to any measurable extent, even though it may show respiration. Once an assimilating cell is destroyed and a false balance of the systems established, even if they may not be inactivated, any net effect of a complex chemical reaction is likely to be small. When oxygen production by isolated chloroplasts is measured in the presence of leaf extracts the pressure of oxygen obtainable is only 1 mm. of mercury. In the living plant the pressure of oxygen reached is several hundred mm. of mercury. In the dark it has been shown that the oxygen is taken up again when chloroplasts are suspended in leaf extract. The speed of reoxidation rapidly increases with increase in oxygen pressure; thus the balance is reached under the present experimental conditions at

1 mm. pressure. But in the living cell the final product of reduction appears as carbohydrate which is but slowly broken down in respiration, hence a high pressure of oxygen can accumulate.

This subcellular evolution of oxygen, then, does not represent normal assimilation and very probably has no direct connexion with carbon dioxide. Yet the activity of the system is relatively high and oxygen as a photochemical product is a characteristic of photosynthesis in green plants.

The most suggestive view is to regard the chloroplast as containing a mechanism, the activity of which can be measured apart from the living cell, which under illumination simultaneously evolves oxygen and reduces some unknown substance which is not carbon dioxide. This substance is capable of rapid reoxidation, being the converse of reaction (3)



Organisms are known which can assimilate  $CO_2$  in the dark while oxidizing inorganic compounds, that is, during the progress of a reaction of the type of (5). Thus it is proved that  $CO_2$  can be reduced by living cells when free oxygen is present quite independently of light and chlorophyll derivatives. So that reactions (3) and (5) provide, qualitatively at least, a means of carbon assimilation; the net production of oxygen in the process will then be a function of the  $CO_2$  reduced. This type of process will obviously have a low efficiency. The substance  $A$  in the chloroplast is, however, assumed to be of the type of a respiratory catalyst. This mode of linking assimilation with a part of respiration may give a high efficiency at low light intensities. The autotrophic anaerobes, which have no possibility of carrying out reaction (5) directly, obtain their energy by a system similar to the green plant. The oxygen, however, never appears as such because it is removed by hydrogen donators in the growth medium, and when these are fully oxidized assimilation ceases.

This hypothesis acknowledges the reduction of  $CO_2$  as being a rather general phenomenon in organisms independently of photosynthesis, and yet it can explain the nature of a similar type of pigment system in the green plant and in autotrophic anaerobes for utilizing radiant energy.

Part of this work was carried out during the tenure of a Beit Memorial Fellowship. I wish to thank Sir Frederick Hopkins, O.M., Professor D. Keilin and Mr G. E. Briggs for their kind help and criticism during the writing of this paper.

## SUMMARY

1. Haemoglobin has been used in a spectroscopic method of measuring both the appearance and disappearance of small quantities of oxygen.
2. Cell-free suspensions of chloroplasts have been obtained from the leaves of various angiosperms by grinding in isotonic sucrose solutions.
3. The observations of earlier workers, that chloroplasts will produce molecular oxygen apart from the living cell, have been confirmed by an independent method, and the quantities of oxygen evolved under different conditions have been measured.
4. The chloroplasts after removal from the cells only evolve oxygen in light when in the presence of extracts of leaves or certain ferric salts, and do not evolve oxygen from carbon dioxide.
5. In particular the chloroplasts will cause a photoreduction of ferric oxalate to ferrous oxalate and oxygen; the oxygen obtained corresponds to the iron reduced.
6. The illuminated chloroplasts in the presence of leaf extracts evolved oxygen up to a pressure of 1 mm. mercury; in the presence of ferric oxalate a pressure of 4 mm. of mercury can be reached.
7. It is concluded that a system can be removed from the cell of a green plant which will convert light energy into a measurable amount of chemical work. That molecular oxygen is produced in these circumstances, places the system in a category unique among other chemical systems.

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# The submicroscopic structure of dental enamel

By J. THEWLIS

*Physics Department, National Physical Laboratory,  
Teddington, Middlesex*

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[Plate 2]

## 1. INTRODUCTION

The use of the microscope combined with various techniques adapted to the histological examination of enamel has revealed many structural details, but nevertheless there is no generally agreed upon definition of what is normal and what is pathological in enamel. Such being the case, any further information about the structure of enamel should be useful.

A well-known limitation of the microscope is that by its use it is impossible to resolve objects the dimensions of which are small compared with the wave-length of the light employed. By the use of X-ray diffraction methods, however, the relative positions of the constituent atoms or molecules of a substance may often be determined with comparative ease. But as Bragg (1937) has pointed out, the properties of many materials appear to depend to a large extent on the behaviour of aggregates, the dimensions of which are intermediate between microscopic and atomic. It is with such submicroscopic aggregates in enamel that the present paper is concerned.

Chemical analysis has shown that about 98% by weight of human enamel consists of inorganic material and it is now generally recognized that this is present largely, if not entirely, as apatite, chiefly in the hydroxy form (Bowes and Murray 1935), the crystallites of which have been shown by the author to be arranged in a definite way and not distributed at random. It was found (Thewlis 1934) that the hexagonal axes of the crystallites tended to be parallel to one or both of two directions (known technically as fibre-axes and labelled (i) and (ii) respectively) which made an angle of about  $30^\circ$  with each other and were inclined to the tooth surface. The majority of the crystallites were parallel to fibre-axis (i) and variations in the degree of perfection of orientation were noted.

Later work showed that these results did not provide a complete description of the crystalline orientation in the enamel, and a relationship was sought for between the physical and biological units of the enamel structure, i.e. between the directions of the crystallite axes and the arrangement of the enamel prisms.

Such a relationship has, in fact, been found to exist and its nature has been examined by X-ray diffraction and photomicrographic methods. From the observed results it has been found possible to describe to some extent the submicroscopic structure of enamel. Only human deciduous enamel has been studied in the present paper but it is hoped to publish shortly the results of investigations on the enamel of other animals.

## 2. EVIDENCE OF A RELATIONSHIP BETWEEN CRYSTALLITE DIRECTION AND PRISM ARRANGEMENT

From the X-ray diffraction photograph of a portion of enamel it is possible to say to what extent the axes (i.e. the hexagonal axes) of the crystallites lie parallel to the fibre-axis, and also to tell whether one or more fibre-axes are present. From the photomicrographs of the same portion of enamel the degree of regularity of the prism arrangement may be assessed. Comparison with the degree of parallelism of the crystallites will then show whether the arrangement of the crystallites is connected with that of the prisms.

### (a) *X-ray examination*

X-ray diffraction photographs were taken of longitudinal sections of teeth, of thickness 0.1–0.2 mm., and the arrangement previously described (Thewlis 1935) was used in which the X-ray beam passes straight through the tooth section at the point to be examined, a photographic plate being set up parallel to the section to receive the diffracted beams. A fine beam of X-rays was used of diameter 0.5 mm. where it struck the section. Each X-ray photograph was therefore due to a cylinder of enamel of height equal to the thickness of the section and of diameter 0.5 mm. (see fig. 1).

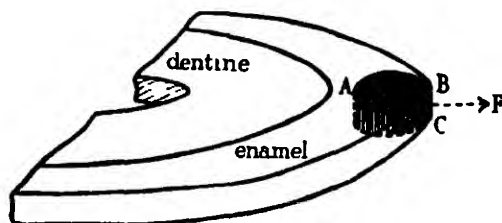
In the previous work three types of enamel photograph were recognized, and termed A, B and C respectively. Similar types of photograph have been observed in the present investigation and their significance is briefly described below.

*Class A.* The crystallite axes in the cylinder of enamel examined are fairly accurately parallel to the fibre-axis (represented in fig. 1 by the arrow *F*), being inclined to it at angles ranging from 0 to about 15°.

*Class B.* Roughly half of the crystallite axes are inclined to the fibre-axis at angles ranging from 0 to about  $15^\circ$ , the rest being inclined at angles from  $15$  to  $70^\circ$  or more.

*Class C.* The crystallite axes are inclined to the fibre-axis at angles ranging from 0 to well over  $15^\circ$ . In most cases the range is from 0 to about  $40$  or  $50^\circ$ , although ranges of  $0$ – $90^\circ$  (corresponding to random orientation) have been observed.

The different classes merge into each other and border-line cases are often observed. These have, however, been omitted from consideration.



$A-B = 0.5$  mm.,  $B-C = 0.1-0.2$  mm.,  $F$  = fibre-axis direction

FIG. 1. Diagram of a tooth section, in which the shaded portion represents the amount of enamel examined at one time.

### (b) Microscopic examination

The method finally adopted for the microscopic examination was to clear a tooth section in xylol and obtain from each side of the section photomicrographs of each portion of enamel already examined by X-rays, i.e. two photomicrographs were taken of each piece of enamel examined by X-rays. A magnification of 120 diameters was employed.

The diversity of the prism arrangement found in the various "areas" of enamel was great, but for the purposes of comparison with the X-ray results these areas were divided into three groups according to the degree of regularity of the arrangement.

### (c) Comparison of photomicrographs with X-ray photographs

Considerations of size show that many crystallites are contained in one prism, so that the prisms and crystallites are not in any sense identical.

The width of a prism of human enamel seems to range in general from  $2$  to  $5\mu$  approximately. The average size of the apatite crystallites on the other hand, estimated from X-ray diffraction photographs, is about  $0.3\mu$  (Bale, Hodge and Warren 1934). A cross-section of a prism will therefore include about 50 or 100 crystallites and thus in  $1\mu$  of a prism's length there will be some hundreds of crystallites.

If, now, on comparing the photomicrographs with the X-ray photographs it is found that irregular prisms are associated with irregularly arranged crystallites (i.e. with class C photographs), and that regular prisms are associated with regularly arranged crystallites (class A photographs), it may reasonably be concluded that the direction of each individual prism bears a constant relationship to the general direction of the axes of its constituent crystallites.

In order to effect such a comparison twenty-four temporary teeth (nineteen molars and five incisors) have been examined, the number of enamel areas investigated being seventy-five. The result of this examination is shown in Table I.

The term "regular prisms" signifies that the prisms in question are straight, and are parallel within about 5 or 10°. Prisms for which the angular variation is not more than about 30 or 40° are classed as "moderately regular", and these prisms may sometimes be slightly wavy in form. Where the angular variation is more than about 40° the prisms are classed as "irregular". The course of such prisms is often markedly wavy and in extreme cases the prisms may even present a criss-cross appearance. As with the X-ray photographs borderline cases are not infrequent, and here again they have been omitted from consideration.

Photomicrographs showing the various kinds of prism arrangement are reproduced in fig. 2, Plate 2.

TABLE I

Arrangement of enamel prisms	No. of areas examined	No. of photographs of class			Percentage of photographs of class		
		A	B	C	A	B	C
Regular	30	14	14	2	46.5	46.5	7
Moderately regular	33	11	17	5	33	52	15
Irregular	12	0	5	7	0	42	58

It will be seen from Table I that regular prisms are not always associated with regularly arranged crystallites. On the other hand, however, no class A enamel is found where the prisms are irregular. There is, therefore, some reason for supposing that a relationship may exist. Observations on the enamel of teeth other than human deciduous teeth support this, for in fourteen areas with irregular prisms so far examined, no class A enamel has been found.

Additional support is also lent by observations made on the number of fibre-axes present in a given area. It has been seen above that either one



or two fibre-axes can occur in human enamel and Table II gives the result of a comparison of the prism arrangement with the number of fibre-axes found in the present series of observations. The terms "single orientation" and "double orientation" refer to the presence of one and two fibre-axes respectively.

TABLE II

Arrangement of enamel prisms	No. of areas examined	No. of photographs showing		Percentage of photographs showing	
		Double orientation	Single orientation	Double orientation	Single orientation
Regular	29	25	4	86	14
Moderately regular	33	17	16	52.5	48.5
Irregular	11	4	7	36	64

It will be seen from this table that in enamel with straight parallel prisms there is a marked predominance of double orientation. Now, if the crystallite direction were connected with the prism direction, this double orientation which is clearly distinguished in the case of regular prisms, would tend to be masked in the case of irregular prisms owing to the ever-changing prism direction (and hence crystallite direction). Consequently the proportion of enamel areas which appear to show single orientation should rise with increasing prism irregularity. Table II shows that there is a definite tendency for this to occur.

It appears then, that the directions of the prism and its constituent crystallites may bear a relationship to each other. The nature of this relationship will be dealt with below, but in the meantime the fact must not be overlooked, that regular prisms are sometimes associated with an irregular arrangement of crystallites.

### 3. THE NATURE OF THE RELATIONSHIP

If the direction of a prism bears a constant relationship to the general direction of the axes of its constituent crystallites then, in areas where the prisms are straight and parallel or nearly so, the angle between the prism direction and the fibre-axis will have a constant value.

This angle has been measured in the case of twenty-eight such areas, taken from six teeth, and as will be seen below, a fairly constant value was, in fact, obtained.

*(a) Experimental method*

As already mentioned one X-ray photograph and two photomicrographs were taken of each enamel area examined. The position of the tooth section relative to the X-ray plate was obtained from a photograph of the tooth in position on the X-ray camera, the X-ray beam being replaced by a beam of light for this purpose. The position of the tooth section relative to the photomicrograph was also obtained in each case so that, in effect, the position of the X-ray plate and hence of the fibre-axis, relative to the photomicrograph, was known.

The average prism direction in both photomicrographs of each enamel area was determined from measurements in eight places and the angle between this direction and that of the fibre-axis was thereby obtained. In some cases where double orientation occurred the position of the second fibre-axis, i.e. that which usually corresponds to a minority of crystallites, could not be determined with certainty, and in these cases no value for the angle between prism direction and second fibre-axis was obtained.

*(b) Results*

The values of the angle between the prism direction and the fibre-axis, obtained from the above measurements, are given in Table III in which they are grouped according to their magnitudes.

TABLE III

Values of angle between prism direction and

Fibre-axis (i)	Fibre-axis (ii)
-4°	31°
-2	35
1, 1, 1	38, 38, 38
2, 2, 2	39
3	40, 40
4	44
6, 6, 6, 6, 6	45
7, 7, 7, 7	
9, 9, 9	
10	
12, 12	
13, 13	
21	

Neglecting the value of 21°, noted in column 1 of this table, which will be considered later, it can be seen that the two sets of measurements are grouped fairly closely about the mean values of 6 and 39°. It may there-

fore be said that the prism direction makes angles of approximately 5 and 40° with the directions of fibre-axes (i) and (ii) respectively.

The fibre-axes are found in all but two cases to point to that side of the prism remote from the tip of the nearest cusp, the direction being taken away from the tooth, as shown in fig. 3. The values corresponding to these two cases are given negative signs in Table III.

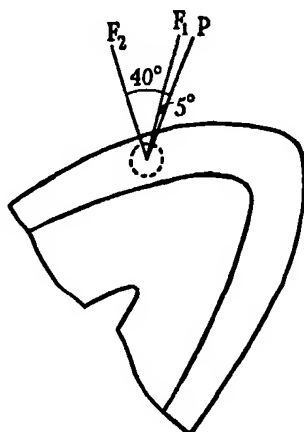


FIG. 3. Diagram of a tooth section showing the relationships of the prism and fibre-axis directions to each other and to the tooth.  $P$  = prism direction,  $F_1$  = direction of fibre-axis (i),  $F_2$  = direction of fibre-axis (ii).

The occurrence of a value of 21° may now be understood, for if fibre-axes (i) and (ii) were both present, with an equal number of crystallites parallel to each axis, and if, as often happens, the X-ray reflexions due to the two sets of crystallites overlapped, then an X-ray photograph corresponding apparently to single orientation would be obtained. The angle between the fibre-axis and prism direction would then appear to be the mean of 5 and 40°, i.e. 22.5°. Presumably this is what has happened in the present case.

#### 4. THE SUBMICROSCOPIC STRUCTURE OF ENAMEL

##### (a) *The nature of the interprismatic substance*

The interprismatic substance cannot be examined directly by X-rays since all the material in the path of the X-ray beam contributes to the X-ray photograph. It is, however, generally recognized to be mostly inorganic in character, and Keil (1936) has shown that it is optically birefringent and crystalline.

If a type of orientation were present in the interprismatic substance which did not exist in the prism material, then a corresponding fibre-axis would be observed on all the X-ray photographs. Two fibre-axes have indeed been noted and the possibility therefore arises that one of them is associated with the interprismatic substance and the other with the prism material. In this case, however, the two groups of crystallites corresponding to the two fibre-axes would be present in approximately constant proportions, whereas in actual fact the 40° group is sometimes entirely absent and is sometimes present to the same extent as the 5° group.

Consequently it may be concluded that no type of orientation is present in the interprismatic substance which is not also present in the prism material. The degree of perfection of the orientation is, of course, not necessarily the same in the interprismatic substance as in the prism material, and the relative extent to which the 5 and 40° groups are present in the two cases may likewise not be identical.

#### *(b) The crystallite arrangement*

(i) *The nature of the arrangement within the prism.* From observations on areas with regular prisms (see Table II) it appears that two groups of crystallites are generally present in a given area of enamel, although sometimes only one group is found. The presence of these two groups may perhaps be regarded as usual in human deciduous enamel, since as stated above, the double orientation is masked when the prisms are not regular.

It is not possible to say at once whether the two sets of crystallites occur in one prism or whether each prism contains crystallites oriented in one direction only. Whatever factor it is, however, which affects the way in which the crystallites are laid down to form a prism, it is not unreasonable to assume that neighbouring prisms are affected in the same way. Moreover, W. J. Schmidt (1937) has published a photomicrograph of the enamel of an elephant's tooth, taken with polarized light, which seems to show that in this case at all events two different arrangements are present in the same prism.\* Further, a series of X-ray photographs, taken along the same set of prisms from the amelo-dentinal junction outwards, shows that the type of orientation sometimes changes (e.g. from single to double) even though the prism arrangement remains the same. Unless it is possible for

\* W. J. Schmidt and his pupils A. Keil and M. Harders-Steinhauser have examined teeth extensively by the polarizing microscope. Their results, some of which have been repeated by the author, are consistent with the X-ray results given in the present communication and may be explained by them in some cases. It is proposed to deal with this aspect of the work in a future paper.

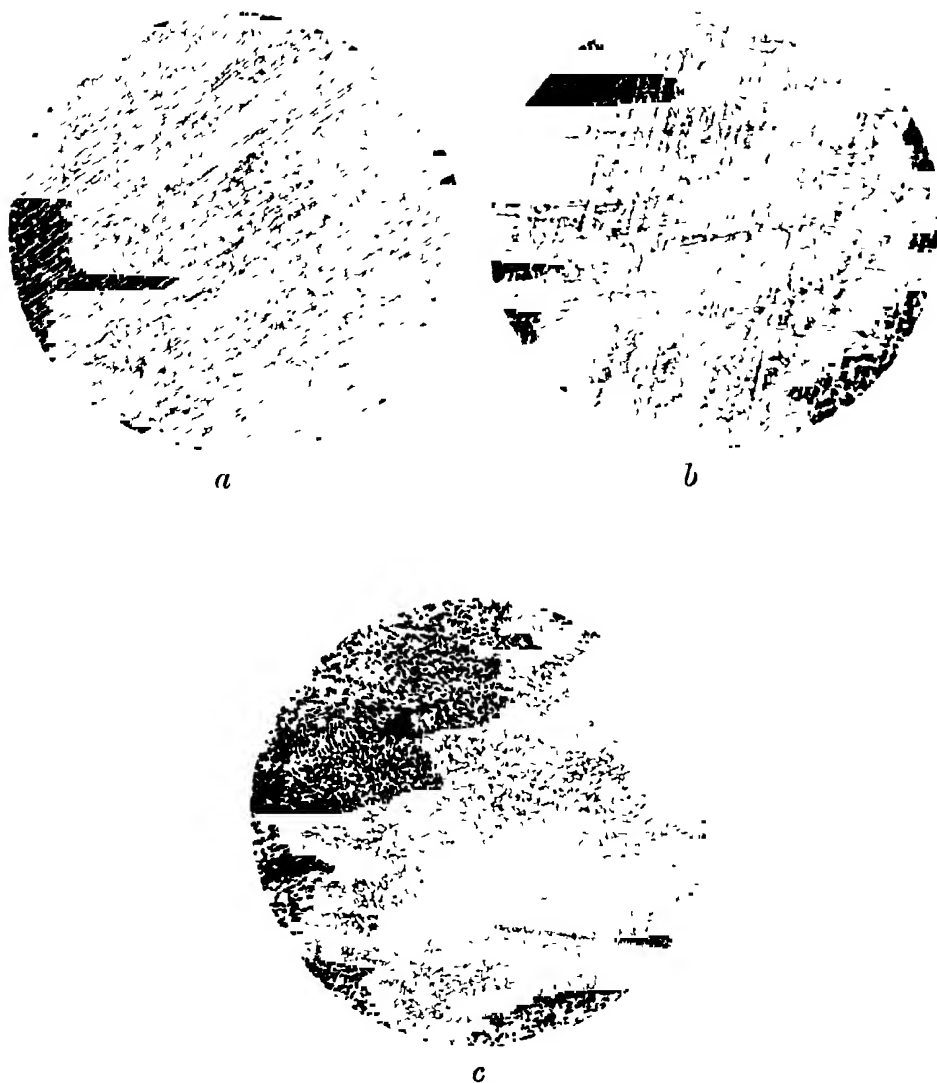


FIG. 2. Photomicrographs showing the various kinds of pinus arrangement ( $\times 120$ ).  
(a) regular, (b) moderately regular, (c) irregular.



the two groups of crystallites to be present in the same prism it is difficult to see how such a change can be accounted for.

Consequently it may be assumed that the two groups of crystallites are in fact contained within a single prism.

(ii) *The degree of perfection of the arrangement.* It might have been thought that the interprismatic substance could account in some way for the observed variations of crystalline arrangement, but consideration shows that this is not likely. An estimate of the amount of interprismatic substance present in enamel, made from measurements of the relative areas of interprismatic substance and prism in photomicrographs of transverse sections, gave a value of not more than about 25 % by volume, and variations of crystalline arrangement which are restricted to this amount of material are not capable of explaining the wide differences observed.

Owing to the relatively small amount of interprismatic substance present, nothing can be said regarding the perfection of the crystalline arrangement in this substance, but from the observations recorded in Table I some information may be gained about the arrangement of the crystallites within the prisms.

It will be seen from Table I that X-ray diffraction photographs of class A, B and C are obtained from areas with regular or moderately regular prisms, whereas only photographs of classes B and C are obtained from areas with irregular prisms.

The presence of class A enamel in areas with regular or moderately regular prisms is what would be expected if, throughout such areas, the average crystallite direction within each prism made a fairly constant angle with the prism direction, the degree of perfection of orientation being high. The presence sometimes of class B or even of class C enamel in similar areas might arise from one of two causes. Either the degree of perfection of orientation may vary within individual prisms or the average direction of the crystallites may vary from prism to prism.

A series of X-ray photographs taken, as before, from the amelo-dentinal junction outwards, shows that the class of enamel sometimes changes even though the prism arrangement remains the same. If the degree of perfection of orientation within individual prisms were not capable of alteration along the length of the prism such a change in enamel class would be difficult to explain. It may therefore be assumed that the degree of perfection of orientation does vary within individual prisms for the cases in question.

The relationship between prism and crystallite direction previously discussed will account for areas with irregular prisms giving rise to class C

photographs. The irregularity of the prism arrangement will cause an apparent low degree of perfection of orientation whether the degree of perfection in individual prisms is, in fact, high or low. The obtaining of class B photographs from such areas might mean that in these cases the degree of perfection of orientation in individual prisms is good and that an appreciable number of the prisms lie in some part of their course parallel to the same direction.

(c) *Discussion of the structure*

It has been concluded above that the apatite crystallites of which enamel is largely composed are so arranged within individual prisms that their hexagonal axes may make, on the average, angles of 5 and 40° with the prism direction. Sometimes the 40° group of crystallites is entirely absent but usually both groups are present, the 5° group predominating.

It has also been seen that the type of arrangement of the crystallites in the interprismatic substance appears to be the same as that in the prisms, the two groups of crystallites being, however, not necessarily present in the same proportions in the two cases.

It has been found too that the degree of perfection of the arrangement may vary within individual prisms (nothing can be said about the interprismatic substance) thus giving rise to the three types of X-ray photograph obtained, and it may be of interest to represent the results diagrammatically.

Fig. 4 illustrates the structure of a piece of class A enamel with double orientation of the most usual type.

Two prisms with their organic sheaths and the interprismatic substance are drawn. The organic sheath has been cut away from one prism so as to reveal the arrangement of the crystallites, which are represented in the figure by short lines signifying the crystallite axes. These lines are arranged in rows, in order the more easily to illustrate the relative numbers belonging to the 5 and 40° groups. This is, of course, diagrammatic, the actual positions of the crystallites, as distinct from their directions, not being known.

Fig. 5 illustrates diagrammatically the structure of a section of one of the enamel prisms represented in fig. 4, the section being taken through the middle of the prism, and as before only the axes of the crystallites are shown. The structures of the prisms in class B and C enamel are represented in a similar fashion in figs. 6 and 7 respectively.

It has been noted above that the presence of a high degree of perfection of orientation or of double orientation within individual prisms is masked



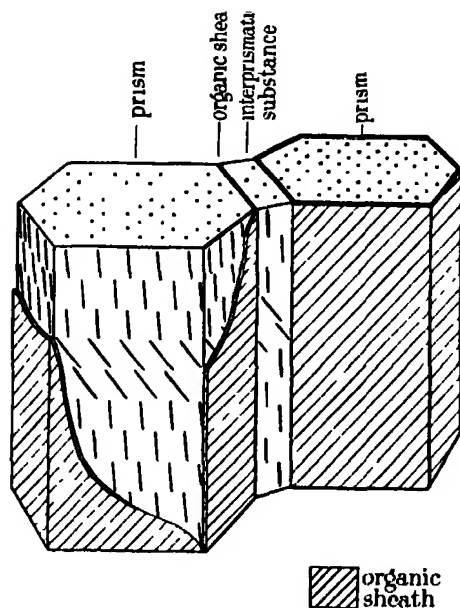


FIG. 4. Diagram illustrating the submicroscopic structure of enamel.  
The crystallite axes are represented by short lines.

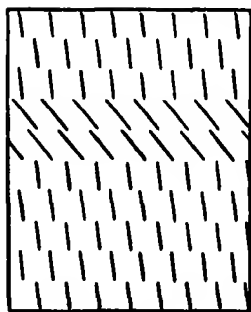


FIG. 5

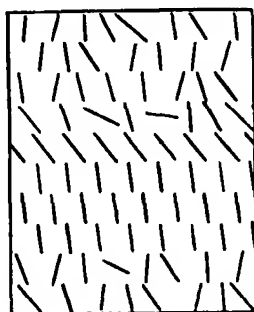


FIG. 6

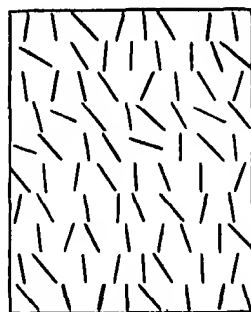


FIG. 7

FIG. 5. Diagram showing the arrangement of crystallites in a prism of class A enamel.

FIG. 6. Diagram showing the arrangement of crystallites in a class B prism.

FIG. 7. Diagram showing the arrangement of crystallites in a class C prism.

when the prism arrangement is irregular. Consequently the fact of obtaining a class B or C photograph or a photograph showing single orientation does not give any useful information about the submicroscopic structure of the enamel unless the corresponding prism arrangement is also known. Where this is known, however, the types of structural variation discussed above may well prove to be of significance, especially if considered together with the results of other methods of X-ray examination and with chemical, histological and clinical evidence.

As mentioned earlier, work is proceeding on the structure of the enamel of teeth other than human, and it may be of interest to note that the general arrangement of crystallites in this enamel appears to be similar to that in human enamel. Similar variations in the degree of perfection of crystallite orientation occur, but the results so far obtained suggest that the actual relationship between crystallite direction and prism direction is not quite the same in different animals.

The above work forms part of an investigation being carried out on behalf of the Dental Disease Committee of the Medical Research Council, to which the author is indebted for permission to publish.

He is also indebted to Lady Mellanby and Mr G. H. Payne, her technical assistant, who supplied and prepared the specimens, to Professor W. L. Bragg for helpful discussion, to Dr G. W. C. Kaye for his interest and to Mr J. A. G. Smith who prepared all the photomicrographs and otherwise assisted in the experimental work.

## 5. SUMMARY

From observations made on photomicrographs and X-ray diffraction photographs of human deciduous enamel, the submicroscopic structure of such enamel has been studied.

It has been found that each enamel prism is composed of a multitude of crystallites of apatite which may be divided into two groups, so arranged that their hexagonal axes tend to make definite angles with the direction of the prism. The values of these angles are approximately 5 and 40° and the crystallite axes are inclined so as to point to that side of the prism remote from the nearest cusp, the directions being taken away from the tooth.

In general both the above groups of crystallites are present in the prism, with the 5° group predominant, although occasionally both groups are equally represented. Sometimes, however, the 40° group is entirely

absent. In some cases variations in the degree of perfection of the crystalline orientation within individual prisms may occur, thereby giving rise to the three types of X-ray photograph observed. In other cases these different types of photograph may be due to variations in prism regularity.

The interprismatic substance, like the prism material, usually contains the 5 and 40° groups of apatite crystallites, but nothing can be said as regards the variation in degree of perfection of the crystalline orientation.

The submicroscopic structure of enamel in certain cases has been discussed and illustrated diagrammatically.

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## The effect of X-rays on the glucose and hexose-phosphate glycolysis of tumour tissue

BY BARBARA ELIZABETH HOLMES, PH.D.

*From the Biochemical Laboratory, Cambridge, and the Strangeways Research Laboratory, Cambridge*

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The clinical approach to questions concerning the effect of X- and  $\gamma$ -rays on cells has, of necessity, been highly empirical. By methods of trial and error, in fact by experience, it has been found that certain tissues are more easily injured by radiation than others, certain types of tumour more responsive than others to treatment by irradiation.

The experiments described here and in previous papers (Holmes 1933, 1935) on the effects of irradiation upon cell metabolism have also been conducted on empirical lines. They represent simply an attempt to carry the work done by others on the lethal effect of the rays one step further

and to describe this effect in terms of cell metabolism. The possibility that detectable differences in the metabolism of different tissues may account for their variability in response to radiation must also be considered, and such experiments may eventually provide the data necessary for investigating this possibility.

Work which has already been published suggests that there is considerable selectivity in the action of X- and  $\gamma$ -rays on metabolic processes and not the general destruction of tissue which might have been expected from earlier views on the subject. For instance it was shown by Crabtree (1932) that irradiation by  $\beta$ - and  $\gamma$ -rays from radium at 37° C caused a diminution of tissue respiration in doses which were too small to effect the anaerobic glycolysis. I showed (Holmes 1933) that the breakdown of glucose by tissue cultures is inhibited by doses of  $\gamma$ -radiation which have no effect on urea and ammonia production and no effect, during the conduct of the experiment, on the movements of the cells in the culture. I suggested that if the oxidative breakdown of glucose were inhibited, the tissue might still be able to rely on the oxidation of protein for its energy supply. Crabtree (1936) found that the inhibition of ammonia formation in tissue slices which is caused by the presence of glucose in the medium, is removed by  $\beta$ - and  $\gamma$ -rays, so that, in the presence of glucose, irradiated tissue slices actually produce more ammonia than the non-irradiated. The experiments to be described in the present paper give further instances of this selectivity.

Histological work has given instances of selective destruction in so far as it seems to be fairly generally agreed that the mitochondria are the first structures to show injury. As an example of extreme insensitivity, on the other hand, there is an excellent example in the purine containing substance with characteristic yellow fluorescence found by Dr W. Jacobson (personal communication—to be published shortly) in certain cells in the pylorus and duodenum. This substance is unchanged by colossal doses (amounting to some 2 million r. units).

One consideration must be borne in mind. It is plain that the selectivity may sometimes be more apparent than real. We may imagine that some enzyme systems are seldom working to capacity and that some molecules are usually present in excess of requirement, whereas others constitute the limiting factors of the respiratory or fermentative processes we are studying. In such cases a given dose of X-rays may appear to have a selective action on the limiting substance or enzyme systems, when, in fact, the action is more evenly distributed.

Interesting work carried out by Mitchell and Allsopp (Mitchell 1937) on

the ultra-violet irradiation of glycyl-tyrosine showed that the compound undergoes a series of reactions involving formation of dihydroxyphenyl compounds. A late step is the breaking of the peptide link and the liberation of glycine. They considered that similar reactions occurred in the protein molecule; thus it might be expected that actual break-up of a protein molecule into smaller fragments might be a late step occurring after a considerable dose of irradiation had been given. It is not known whether the results of  $\gamma$ - and X-radiation would be the same, but it is known from Crabtree's (1932) work with preparations of succin-oxidase and my own (unpublished) observations on purified preparations of peroxidase that these enzymes are relatively insensitive to X- and  $\gamma$ -radiation, so that, as far as we can judge, fragmentation of these protein molecules is not easily brought about by irradiation. The results presented in Table III perhaps afford further evidence for this.

Some mention must be made of the work which has been carried out on the irradiation of simpler chemical substances in aqueous solution. Colwell (1932) showed that  $\beta$ -rays caused the reduction of Fehling's solution, and the reduction of aqueous solutions of methylene blue and nitrates including ammoniacal silver nitrate. A great deal of work has been done by Fricke (1935*a, b, c, d*) who has studied the oxidation of ferrocyanide, nitrite, arsenite and selenite, the decomposition of  $\text{H}_2\text{O}_2$  and many other reactions brought about by X-rays. Fricke is of the opinion that such reactions are brought about by the activation of water molecules by the irradiation (see Fricke 1935*b, c, d*) and subsequent reaction of the activated water molecules with the compound in solution; hydrogen peroxide is frequently produced but not when pure water alone is irradiated. He points out that the amount of nitrite oxidized is independent of the concentration of nitrite in concentrations from 0.05-100 millimoles per 1000 c.c. Gray (personal communication, for which I am grateful) has obtained similar results when studying the reduction of methylene blue by X-rays, so that this also seems to depend on the activation of water molecules in the first place. It is plain that in concentrated solutions or solutions of large molecules where the possibility of direct hits on the molecules is greater, more attention must be paid to the probable effect of direct hits, whereas in dilute solutions the activation of the water may be the chief factor. It is easy to see that this idea of water activation is extremely important when the events following X-radiation of tissues are being considered. It must be pointed out however that changes can still be brought about when substances are not in solution, although the dosage required may be greater.

Thus Becker (1933) showed that if oxy-proline was irradiated by X-rays he obtained a substance giving a marked absorption band in the ultra-violet. He irradiated the oxy-proline in solution, in the solid state in the presence of oxygen and in the solid state in the absence of oxygen, and obtained this same substance in all conditions. When the irradiation was carried out in solution the absorption was more marked, and a further band developed, suggesting that the reactions had proceeded more quickly. It is especially interesting to note that if proline was used instead of oxy-proline the reaction was apparently much slower since the absorption was never so marked, and one of the typical bands was never obtained.

Spicer (1935) showed that reduction of ferric chloride by  $\alpha$ -particles occurred when the salt was in aqueous solution, in dry ethereal solution and in solid form. The reduction, by the doses of  $\beta$ - and  $\gamma$ -rays employed by him, did not occur in aqueous solutions but did in anhydrous ethereal solutions. Here the possibility must be considered that the molecules of the ether were first activated and that these brought about the reduction.

We cannot assume that all the observed phenomena are brought about by activated water or other solvent though there is evidence that some of them are. In tissue cells, which contain about 20% solid matter, it is likely that we shall have to consider reactions brought about through activated water and also some which are the result of direct irradiation of the solids.

The biological work would gain enormously in interest if we could relate it with work on simpler systems. This is not easy, as we must suppose that the physical condition of molecules inside the living cell is likely to affect their response to irradiation. Thus they may be exposed to the action of activated water or protected from it by their position in the cell, they may be free in solution or attached to other large molecules, they may possibly be induced by enzyme systems to break down in an unexpected manner as a result of absorbing energy from irradiation.

We have already some evidence that the physical conditions under which irradiation of tissues is carried out affect the results. For instance Crabtree (1935) has shown that if irradiation ( $\beta$ - and  $\gamma$ -rays from radium) is carried out at 37° C, the respiration of the tissue is first affected (though sometimes only by large doses) while the anaerobic glucose breakdown is unaltered. If, however, the irradiation is carried out between 0 and 10° C the anaerobic glycolysis becomes much more sensitive and is strongly inhibited by doses which leave the respiration unaltered; the respiration is much less sensitive to irradiation when the temperature is low.

Crabtree and Cramer (1934) have also shown that the susceptibility of

tumour cells to irradiation is affected by temperature and by the presence or absence of oxygen (as well as by the presence of chemical substances such as cyanide, fluoride and iodoacetate, which have a marked effect on cell metabolism).

It has long been believed that rapidly proliferating tissues are more susceptible to irradiation than those in a "vegetative" state. Tansley, Spear and Glücksmann (1937) have shown that cells irradiated shortly before division are likely to break down subsequently when mitosis is attempted. This may be because division is a process requiring peculiar metabolic activity or a special alteration in the physical state of protein molecules which an irradiated cell cannot accomplish, or it may be that the rearrangement of cell contents and the alteration of the physical state of the protein molecules which we know occur during mitosis are responsible for the varying degrees of sensitivity to irradiation. Such problems will become more approachable when we know more of the metabolic activities accompanying mitosis.

To consider simpler problems first, it is of obvious interest to irradiate extracted or partly purified enzymes and co-enzymes and to compare their susceptibility in this state with their susceptibility in the living cell. By doing this we can make a first step in the direction of deciding to what extent the observations made on the effects of irradiation of substances in solution can be applied in the understanding of its effects on cells.

In the work described here the whole tumour tissue is irradiated, and the glycolysis measured afterwards in the chopped tissue. Fortunately, it is equally easy to make extracts of the tumour tissue and irradiate them and carry out the same measurements on these extracts. This will form the subject of a later communication.

The X-ray dosage used in these experiments is very large (70,000 r. units approx.), but the changes in metabolism investigated are immediate changes. It is well known that the dose required to produce immediate death of tissue cultures is also very large, and very much greater than that required to produce a delayed lethal effect (see Spear 1930). I have not, therefore, concerned myself as yet with the relation of the dosage required to produce death and the dosage required to produce metabolic changes but in later work attempts will be made to produce delayed metabolic effects by smaller doses. A comparable dose of  $\gamma$ -radiation used in earlier work (Holmes 1933) undoubtedly produced an inhibition of carbohydrate breakdown without noticeably altering the appearance of the cells or the activity of their movements in the cultures (these were *not* dividing cells). It is also very interesting to learn that Crabtree in collaboration with Gray

(1939) has found that the glycolysis of retina is extremely sensitive to irradiation.

#### EXPERIMENTAL

Crabtree (1935) showed that anaerobic glycolysis in various tissues can be inhibited by  $\beta$ - and  $\gamma$ -rays much more readily at 0–10° C than at higher temperatures. Irradiation at 0° C has the further advantage that the low temperature delays degenerative changes in the tissue. These experiments, which were intended as a more detailed investigation of the effects of X- and  $\gamma$ -rays on glycolysis, were therefore carried out at 0° C. The particular problem was to decide whether the glycolysis of the hexosephosphates was affected by the irradiation to the same extent as glucose glycolysis.

Two or three experiments were done with the help of Dr Crabtree and with his apparatus at the laboratories of the Imperial Cancer Research Fund in order that his conditions might be exactly repeated. As I had previously found (Holmes 1937) that tumour brei but not tumour slices could glycolyse hexosediphosphate, brei was used in these experiments instead of slices. The amount of brei which could be used was small, and after the necessary 4 hr. keeping in the ice-chest, the glycolysis, even in the controls, was small.

The Crocker tumour was used, and as explained in the earlier paper (Holmes 1937), this tumour loses its power to produce lactic acid from hexosediphosphate after it has been kept for some hours in the ice-chest, but if pyruvate is added lactic acid is formed once more (confirmed by chemical estimation). This shows that the tissue still forms triosephosphate from hexosediphosphate and can still bring about the dismutation between triosephosphate and pyruvate with the resulting formation of lactic acid. It was thus necessary to add pyruvate in this first experiment; adenylic acid and coenzyme I were added as usual (Holmes 1937, following the suggestion of Boyland). For details of the radium containers and irradiation apparatus Crabtree's paper (1935) should be consulted;  $\beta$ - and  $\gamma$ -rays from the radium are employed. The apparatus was cooled before the tissue was put in and kept in the ice-chest during the irradiation. The control tissue was also kept in the ice-chest (the radium being, of course, well surrounded by lead). After 4 hr. manometric measurements were made of acid production by the tissues, and comparison made between the acid production in glucose and that in hexosediphosphate in the presence of pyruvate, coenzyme I and adenylic acid. It was plain that a dose of  $\beta$ - and  $\gamma$ -radiation which was sufficient to cause at least a 50% inhibition of glucose glycolysis had no effect on the lactic acid formation from hexose-



diphosphate. From these first experiments it appeared that at least the enzymes concerned in the formation of triosephosphate and its dismutation with pyruvate to form lactic acid are relatively insusceptible to irradiation. It was obviously necessary to carry the investigation further, using more and, if possible, more active tissue. Tumour 113 (the "glycogen tumour") was found to be far more suitable than the Crocker for this purpose since it can form lactic acid from hexosephosphate actively without the addition of pyruvate, even after several hours' keeping in the ice-chest. I am grateful to Dr Cramer for suggesting the use of this tumour.

The irradiation was carried out at the Strangeways Laboratory. The tissue, in small stoppered glass bottles, was immersed in ice and water and at a distance of 10.5 cm. from the target of the X-ray tube. Except for 0.05 cm. copper in the tube itself no screening was used, and the dose received was about 710 r. units per min. allowing for the small amount of absorption by the glass of the bottles. The X-ray tube was running at 150 kV constant potential, 7 mA. One and a half hours was found to be sufficient to cause a 50% or greater inhibition of lactic acid formation from glucose when tested later in a Warburg manometer, and this dosage was subsequently used. The control tissue was also kept in ice and water. The tumour to be irradiated was removed from the animal and divided into two fairly equal portions, care being taken to divide "healthy" tissue and any necrotic tissue as far as possible equally between the two. One portion was irradiated and the other kept as control. After irradiation, the necrotic tissue of both portions was removed as far as possible and the live tissue chopped in cooled dishes and measured with a cooled tissue syringe into the manometer flasks. The measurements were made by Warburg's method and were carried out in a bicarbonate buffer in an atmosphere of  $N_2 + 5\% CO_2$  in Warburg manometers. The amount of acid formed was measured in terms of the  $CO_2$  evolved.

These experiments made it clear that a dose sufficient to inhibit glucose glycolysis had a much smaller effect on hexosediphosphate glycolysis. The effect on glucose glycolysis became increasingly marked during the course of the manometric experiments (see fig. 1), (indicating that lapse of time was necessary before the full effect of the dose was demonstrable). In this particular tumour, unlike others that have been tried, the glucose breakdown after a few hours was feeble and the hexosephosphate breakdown still active. The inhibition of glucose breakdown by the irradiation was quite evident in spite of this.

After it had been found that the hexosediphosphate breakdown was

relatively insusceptible to  $\beta + \gamma$ - and also to X-rays, experiments were carried out with hexosemonophosphate (Embden ester).

In this case also it was found that the same dose of X-rays had a small or no effect on hexosemonophosphate glycolysis, thus showing that the process of phosphorylating the monophosphate (Embden ester) to form diphosphate as well as the steps occurring subsequently during the breakdown of diphosphate to lactic acid are not affected by a dose of X-radiation sufficient to have a marked inhibitory effect on glucose glycolysis.

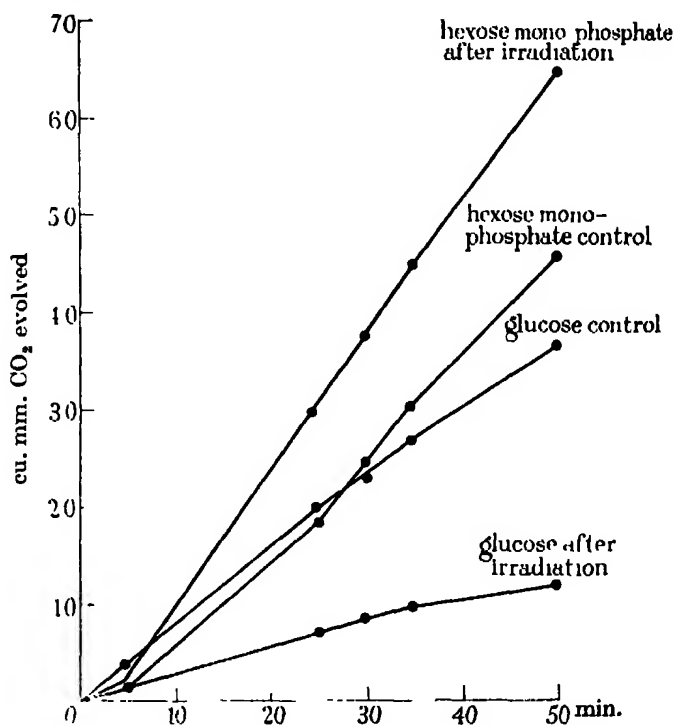


FIG. 1

Fig. 1 represents an experiment carried out with hexosemonophosphate and glucose. In this particular case the irradiated tissue showed a larger hexosephosphate breakdown than the control tissue, but this is not usual and cannot be regarded as significant. The rate of glucose glycolysis towards the end of the experiment illustrated is practically nil and hardly exceeds the acid production expected in the absence of glucose.

The fact that the acid produced from the hexosephosphates was actually lactic acid was confirmed, on several occasions, by chemical estimation of lactic acid at the end of the experiment; examples are given in Table I.

TABLE I. CU. MM. CO<sub>2</sub>. 0.15 G. TISSUE. TEMP. 31° C. HEXOSEPHOSPHATE CONCENTRATION M/30 APPROX.

Duration of exp. min.	Glucose control	Glucose irradiated	Hexose- phosphate control	Hexose- phosphate irradiated	By estimation chemically		Remarks
					Lactic acid control mg.	Lactic acid irradiated mg.	
60	184.0	42.0	41.0	38.0			Irradiation: 4 hr. with radium. Diphosphate used. Crocker tumour
30	53.0	15.0	22.0	21			Diphosphate used
30	25.0	9.0	98	91			Monophosphate used
45	67.0	44.0	105	110			Monophosphate used. Shorter irradiation (1½ hr.)
60			152	131	0.85	0.82	Diphosphate 0.25 g. tissue
90			123	118	0.7	0.65	Monophosphate 0.2 g. tissue
30			116	147	0.56	0.72	Monophosphate
120	57.0	28.0	78*	71*	0.76	0.72	Monophosphate CO <sub>2</sub> : out- put masked by NH <sub>3</sub> production from adenylic acid excess
30	38.0	13	126	91			Monophosphate
120	57	28	90	88			Cori ester
85	56	15	61.5	105			Cori ester (see fig. 1)

These figures represent the total amount of lactic acid present at the end of the experiment; owing to shortage of tissue initial values could not be obtained. Another series of experiments was therefore carried out in which the initial values were estimated and subtracted. These experiments are summarized in Table II; the lactic acid values given represent the amount of the acid formed during the experiment. It can be seen that the chemical estimations entirely confirm the manometric and demonstrate the inhibition of glucose glycolysis (sometimes practically complete) by X-rays and the relative stability of hexosephosphate glycolysis.

It should be pointed out that small-sized Warburgs (total volume 6-7 c.c.) are used, and that the glycolysis figures, though small, represent large excursions on these manometers.

The  $Q\ CO_2$  nomenclature is not used in Table II. Since brei was used and not tissue slices, and since the brei had been kept at  $0^\circ\ C$  for some time and must always have included some necrotic tissue from the tumour, it is felt that use of the conventional nomenclature would not be helpful. The figures given represent the total amount of  $CO_2$  evolved in the time stated, 0.15 g. of brei being used.

The solution of coenzyme I and adenylic acid generally used contained 5 mg. of a coenzyme preparation (kindly supplied by Dr D. E. Green) and 10 mg. muscle adenylic acid per c.c.; 0.4 c.c. were used in the large manometers and 0.2 or 0.3 c.c. in the small. These activators were sometimes added also to the tissues in glucose solutions to see if they could effect glucose glycolysis as well as hexosephosphate glycolysis. Only very small and transient increases of acid production were caused by this addition. Hexosephosphate glycolysis, on the other hand, proceeds only after the addition of these activators.

As pointed out by Boyland, tumour tissue destroys coenzyme I and adenylic acid rather rapidly. The duration of hexosephosphate glycolysis and therefore the total production of lactic acid depends partly on how long a sufficient amount of the activators remains intact. It is not always possible to obtain samples of tumour tissue (even when they are taken, as in this case, from different parts of the same tumour) which destroy the activators at exactly the same rate, and there is, therefore, some variation in the total amount of lactic acid produced from hexosephosphate. It seems that the irradiation does not in any way affect this and the total lactic acid production is sometimes slightly greater in the irradiated tissue and sometimes in the control tissue.

TABLE II. TOTAL GLYCOLYSIS MEASURED AND EXTRA LACTIC ESTIMATED CHEMICALLY

Method	Extra lactic acid from glucose		Extra lactic acid formed from hexosephosphate		Remarks
	Normal tissue	Irradiated tissue	Normal tissue	Irradiated tissue	
Glycolysis measured	0.135 c.c.	0.047 c.c.	0.182 c.c.	0.206 c.c.	Hexosediphosphate
Lactic estimated	0.83 mg.	0.22 mg.	0.92 mg.	1.1 mg.	
Glycolysis measured	0.188 c.c.	0.048 c.c.	0.129 c.c.	0.119 c.c.	Hexosemonophosphate
Lactic estimated	0.9 mg.	0.08 c.c.	spoiled	spoiled	
Glycolysis measured	0.100 c.c.	0.038 c.c.	0.139 c.c.	0.143 c.c.	Hexosemonophosphate
Lactic estimated	0.33 mg.	0.00 mg.	0.78 mg.	0.84 mg.	
Glycolysis measured	0.116 c.c.	0.048 c.c.	0.067 c.c.	0.062 c.c.	Cori ester
Lactic estimated	0.57 mg.	0.20 mg.	0.39 mg.	0.43 mg.	
Glycolysis measured	0.146 c.c.	0.041 c.c.	0.183 c.c.	0.142 c.c.	Cori ester
Lactic estimated	0.7 mg.	0.00 mg.	0.91 mg.	0.75 mg.	
Glycolysis measured	0.109 c.c.	0.045 c.c.	0.145 c.c.	0.145 c.c.	Cori ester
Lactic estimated			0.60 mg.	0.56 mg.	
Glycolysis measured			0.152 c.c.	0.131 c.c.	Hexosediphosphate
Lactic estimated			0.60 mg.	0.56 mg.	
Glycolysis measured	0.115 c.c.	0.034 c.c.			Tissue slices weight 0.6 mg. only (dry)
Lactic estimated	0.26 mg.	0.00 mg.			

## EXPERIMENTS WITH THE CORI ESTER

It is considered that, in the breakdown of glycogen to lactic acid by tissues, the formation of glucose-1-phosphate precedes the formation of the Embden ester (Cori, C., Cori, G. and Colowick 1937). It has been found possible to show the breakdown of glycogen by tumour brei, but the brei will form lactic acid from the glucose-1-phosphate (Cori ester). This, therefore, represents the earliest stage of glycogen glycolysis which it is possible to study at present. I am indebted to Dr and Mrs Cori for a sample of the ester; a further sample was prepared by the method of Kiessling (1938). The amounts of Embden ester contained in these samples are too small to account for the lactic acid production observed, since lactic acid production only proceeds in the presence of a considerable concentration of hexosemonophosphate (the concentration generally used was  $M/30$   $M/50$ ). The first sample contained 5-8% of the Embden ester and the second sample small traces only.

Just as in the case of the Embden hexosemonophosphate it can be shown (see Tables I and II) that the formation of lactic acid is not inhibited by  $1\frac{1}{2}$  hr. irradiation. Since the "Cori" ester is the earliest link in the chain of glycogen breakdown investigated so far, this result confirms and, in fact, includes the results already mentioned. For this reason an experiment with Cori ester was chosen for illustration in fig. 1.

As adenylic acid and coenzyme I have to be added in any case in these experiments, it is impossible to tell whether any destruction of these activators occurs in irradiated tissue. The experiments with the lactic dehydrogenase system in embryo tissue, which are described below, represent an attempt to investigate this possibility.

## LACTIC DEHYDROGENASE SYSTEM

It was reported previously (Holmes 1935) that the reduction of methylene blue by embryo brain tissue in the presence of lactic acid, could be inhibited by  $\gamma$ -radiation. Older embryos, both rat and chick, did not show this effect, but the inhibition was plain in the case of 8-day chick brains and rat embryos at about the 14th-16th day.

Much further work has fully confirmed this, and suggests that the dehydrogenase itself is not so readily damaged as the coenzyme or carrier part of the system. By the addition of enough coenzyme I (approximately 0.5 mg. added to 50 or 100 mg. of tissue in 1.5 c.c. fluid) the activity of the control tissue, which has naturally diminished during the period of the

irradiation, can be considerably increased and the much further diminished activity of the irradiated tissue can sometimes be actually brought back to the level of that in the restored control tissue. This result suggests that the dehydrogenase enzyme itself is not damaged in these cases and that some part of the carrier system is susceptible to irradiation. Other results are not so clear as this, and in these cases, although the irradiated tissue shows a relatively greater re-activation by the addition of coenzyme it still does not show the full activity of the control tissue with coenzyme. It is possible that the addition of still further coenzyme might restore it completely. If it becomes possible to investigate this problem on a large enough scale to decide whether coenzyme I itself is destroyed or whether it is the factor concerned with re-oxidation of the coenzyme (Dewan and Green 1938) the work will be carried out, as it will provide interesting material for comparing results as obtained when using living cells with those obtained when irradiating the partially purified preparations of coenzyme and oxidizing factor.

TABLE III. EMBRYO BRAINS FROM 8-DAY CHICKS IRRADIATED 16 HR. BY  $\gamma$ -RAYS FROM 300 MG. RADIUM AT 0.5 CM. DISTANCE. TIME IN MINUTES TAKEN TO REDUCE 0.2 C.C. 1/1000 METHYLENE BLUE. 0.05 G. TISSUE

Atmosphere during irradiation	Control	Control + coenzyme	Irradiated	Irradiated + coenzyme
Air	90	50	> 240	48
Air	23	9	65	20
Air	41	15.5	105	17.5
Nitrogen	17	11	60	13
Nitrogen	21	17.5	70	21
Nitrogen	20	8	75	19

Estimation of the reduction time of the methylene blue was always carried out in evacuated Thunberg tubes and in the presence of lactate. In the absence of lactate the reduction time was very much increased. Each embryo brain was halved, one half irradiated and the other used as control; about 4 brains were used in each experiment.

The results up to date (Table III) are quoted here as having a possible bearing on the question of selectivity of X-ray effects. In the young embryos the whole lactic dehydrogenase system is very poorly developed, whereas in the older embryos it has become more active, the rate of reduction of methylene blue is greater and the loss of activity on keeping is not nearly so great. (This loss of activity on keeping depends partly on exposure to oxygen, and is noticeably less when the tissue is kept in nitrogen; see Table III.) It therefore seems reasonable to suppose that the effects of

$\alpha$ - and  $\gamma$ -radiation on young embryos are much more noticeable than the effects on older embryos simply because the supply of enzyme and carriers is so small in the young embryos that any destruction is immediately seen. In the older embryos one may imagine that there is a certain margin in excess of requirements so that a small effect is not easily seen.

This is, of course, only a tentative suggestion and certainly not capable of general application. The possibility was considered that the relative sensitivity of glucose glycolysis, for instance, is due to the fact that in 0.2 % glucose the enzymes are working at full capacity and that any loss of activity is thus immediately seen. A comparison was on one occasion made between the tissues in 0.2 % and in 0.025 % glucose solutions, and it was found that although glycolysis was not proceeding nearly as quickly in the tissues badly supplied with glucose, still the inhibiting effect of the irradiation was relatively the same. The inhibiting effect of irradiation on glucose glycolysis is therefore still obvious even when glycolysis is not proceeding at its maximum speed. The results were as follows (Table IV).

TABLE IV. 0.15 G. TISSUE USED. CU. MM. CO<sub>2</sub> IN 1 HR. 50 MIN. AT 31° C

0.2 % glucose		0.025 % glucose	
Control	Irradiated	Control	Irradiated
204.0	62.0	88.0	26.0
	(67 % inhibition)		(66 % inhibition)

There was no falling off in the rate of glycolysis in any of the four samples during the whole course of the experiment.

Here, therefore, it cannot be concluded that the apparent sensitivity of the system is due to shortage of enzyme or possible coenzymes.

I wish to thank Dr Lea of the Strangeways Laboratory for his kind help and the British Empire Cancer Campaign for a personal grant.

#### SUMMARY

(1) The work of Crabtree, showing that  $\alpha$  or  $\gamma$  radiation of tumour tissue, kept at 0° C during the irradiation, can inhibit the activity of the tissue in forming lactic acid from glucose, has been confirmed.

(2) It has been shown that a dose of  $\alpha$  radiation sufficient to inhibit the breakdown of glucose by the tissue, has no effect on the formation of lactic acid from hexose-di-phosphate, hexose-mono-phosphate (Emden ester) or glucose-1-phosphate (Cori ester).



(3) The decrease in activity of the lactic dehydroglucose system in young embryo brains, which is caused by irradiation, can be corrected, completely or partly, by the addition of coenzyme 1 to the tissue.

(4) A short discussion of relevant literature is given.

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## Researches on plant respiration

### V. On the respiration of some storage organs in different oxygen concentrations

By J. K. CHOUDHURY, Ph.D.

*Botanical Department, University of Birmingham*

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#### INTRODUCTION

Respiration in complete absence of oxygen, and the relation of this anaerobic respiration to aerobic respiration, have attracted the attention of plant physiologists for a long time, but the effects of different concentrations of oxygen on the respiration of plant material have been studied very little.

Until 1928 the accepted view of this question seemed to have been based on the work of Stich (1891) and Johannsen (1885), which suggested that little or no change could be observed in the rate of carbon dioxide evolution by higher plants over a considerable range of oxygen concentration in the environment. Later, in 1923, Wurmser and Jacquot found that the respiratory rates of *Laminaria saccharina* increased with the oxygen content of the surrounding sea water. Two years later, however, Hée and Bonnet could not find any direct relationship between the oxygen content of the surrounding water and the rate of respiration of *Elodea* and *Myriophyllum*. It is important to note, as pointed out by Mack (1930), that the experiments of these earlier workers usually lasted only a few hours, and these, as subsequent work has shown, may yield results from which it is possible to draw entirely misleading conclusions.

With the work of Blackman and Parija, published in 1928, there began a new chapter in the study of respiration. These workers found that in apples (Bramley's Seedling) either an increase or decrease in oxygen concentration from about 5% increased the intensity of respiration. This particular percentage (which might vary according to the age of the fruit) has been called the "extinction point of NR" (that is, respiration in nitrogen), when the oxygen available was just sufficient to stop anaerobiosis. As the percentage of oxygen was increased the respiration rate also

increased simultaneously, to reach in 100 % oxygen 1.4 times the rate in air, after a period of 45 hr. This increased rate has been explained by Blackman as due to "pre-glycolytic" reactions which lead to an increased rate of glycolysis. On the other hand, the increase in the respiratory rate due to a decrease of oxygen concentration below 5 % was supposed to be due to "post-glycolytic" reactions. Similar experiments with senescent tropical fruits were made by Singh and his co-workers in 1937 with similar results. In their researches the oxygen concentration for mangoes was found to be 9.2 % at the "extinction point". Mack in 1930 published a paper on the relation of temperature and the partial pressure of oxygen to respiration of germinating wheat. Respiration rates were measured at five different temperatures between 10 and 30° C, and in twelve different oxygen concentrations ranging from 0.6 to 98.3 %. At each temperature, however, the respiration rate increased with an increase in the oxygen concentration, until a "subordinate optimal pressure" of it was attained (varying between 6.3 and 20 % oxygen according to the temperature used). With an increase in oxygen concentration from this "optimal pressure", the rate began to fall to a minimum, after which, with still further increase, a second maximum was gradually attained at the "main optimal pressure" of 90–95 % oxygen. The rate of respiration at the highest oxygen concentration used, 98.3 %, was always lower than that at 90 %.

In 1930 attention was also drawn to this problem by F. F. Blackman with the announcement that "the CO<sub>2</sub> production of plant tissue varies with every alteration of oxygen concentration in the environment". This statement has been found to apply in the cases of some senescent fruits, but how far it holds in general is not known. An attempt has been made here to study the respiratory behaviour of some storage organs, in which carbohydrate is the chief reserve, under a wide range of oxygen concentration varying from zero to 98.6 %.

#### MATERIAL AND METHOD

The plant material used in this investigation consisted of tubers of potato (var. King Edward VII) and Jerusalem artichoke, and roots of carrot and red beet, obtained from the ordinary market supply. In all experiments whole organs were used. Before an experiment the material was washed thoroughly in tap water and carefully dried with clean dry linen. When a sample consisted of potatoes, they were next weighed and placed in the respiration chamber, but in almost all other cases, as a result of experience derived from preliminary experiments, the organs were

sterilized as a precaution against fungal attack by dipping them into 0.2 % mercuric chloride for 2 min. They were then washed thoroughly in running water, dried and weighed. Leaves were removed wherever present and no attempt was made to seal the wounds thus caused, except in four experiments with carrots (Exps. 37-40) in which the wounds were covered with grafting wax.

Each sample was placed on a glass support inside the respiration chamber. The latter consisted of two glass hemispheres with equatorial flange and two polar hook-shaped entrance and exit tubes for passage of gas. The two halves of the chamber were clamped together between two brass rings fixed to an iron stand. The chamber had a capacity of about 3500 c.c. It was held in a large bath, regulated at  $25 \pm 0.5^\circ \text{C}$  by a mercury-toluene gas thermoregulator. A fan-type electric stirrer maintained uniformity of temperature in the bath.

The respiration was measured by the Pettenkofer method during successive 3 hr. periods with the aid of a Blackman air-current commutator. All precautions were taken to ensure a uniform rate of bubbling of gas through the absorption tubes, the passage of the gas through the apparatus being at the rate of about 2000 c.c./hr. In each experiment no record was kept of the respiration during the first 90 min., during which time air deprived of its carbon dioxide was drawn through the apparatus at the usual rate. This time was allowed, and found sufficient, to scavenge out all carbon dioxide from inside the apparatus.

The respiratory activity of the various organs was examined in a series of oxygen-nitrogen gas mixtures containing the following percentages of oxygen: 3.5, 6.2, 6.7, 7.9, 10.7, 24.4, 25.5, 40, 45.6, 51.6, 69.3 and 79.9. In addition to these "pure" nitrogen and oxygen were employed as well as atmospheric air (20.8 % oxygen). These gas mixtures were supplied in cylinders by the British Oxygen Company and were analysed by the Haldane gas analysis apparatus before use. When cylinder nitrogen was used it was always passed first through alkaline pyrogallate solution and then through moist soda-lime to get rid of any traces of oxygen and carbon dioxide that might be present in the cylinder. After such treatment, estimations with Haldane's gas analysis apparatus failed to show any measurable quantity of these impurities. In cases of other gas mixtures, although carbon dioxide was absent or negligibly small (well within experimental error) care was always taken to pass the gas through moist soda-lime before it entered the chamber. Pure cylinder oxygen was found on analysis to contain 98.6 % oxygen.

In each experiment the respiration of a sample of tubers or roots was

examined over a continuous period of from 7 to 14 days, the respiration rates throughout being expressed as mg. carbon dioxide evolved per hour per 100 g. fresh weight of material at the beginning of the experiment. For the sake of clearness and brevity the course of respiration is shown in graphical form rather than by recording the detailed numerical results. Only sixteen experiments out of some sixty are recorded in detail, but numerical data of some of the remainder are given in tables to bring out certain observations made in the text.

## RESPIRATION RECORDS

### (1) *Potato*

The results of Exps. 2, 3, 14, 15 and 16 with potato tubers are shown in serial order in the first five figures of the text. In fig. 1 is shown the normal course of respiration in air and in fig. 2 is shown the effect of "pure"

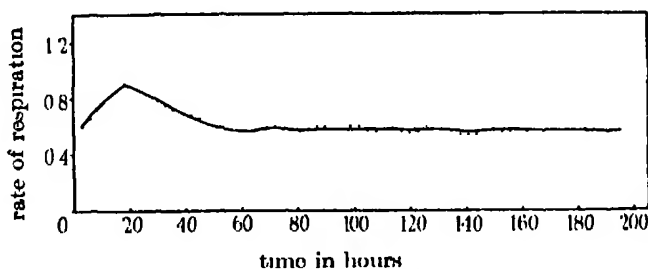


FIG. 1. Course of respiration of potato tuber in air, Exp. 2.

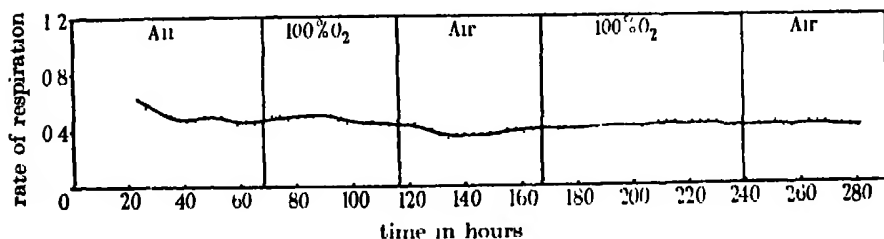


FIG. 2. Potato, Exp. 3.

oxygen on this course. In another experiment (Exp. 5) an attempt was made to keep the potatoes in "pure" oxygen throughout the experimental period of 189 hr., except for an intervening period of 72 hr., when oxygen was replaced by air. The respiratory rate, however, did not show any deviation from the normal course. In fig. 3 are shown the effects of

various oxygen concentrations on the same sample of potatoes. Most of these concentrations have also been tried individually, but in no single case was any deviation from the normal course observed.

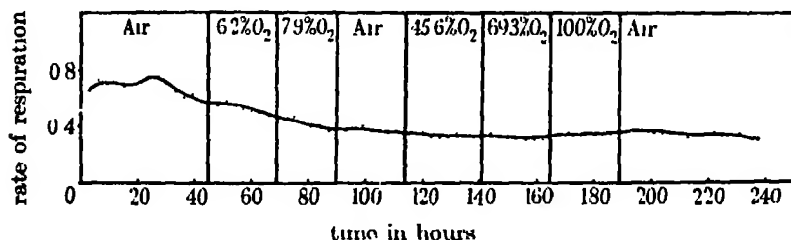


FIG. 3. Potato, Exp. 14.

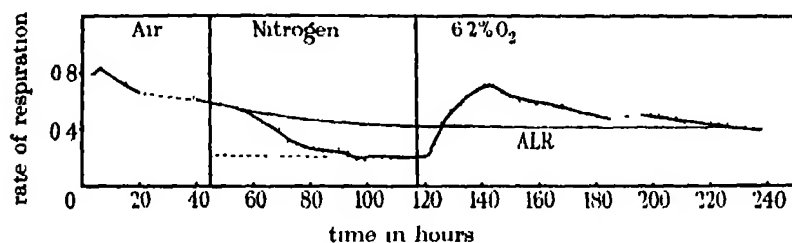


FIG. 4. Potato, Exp. 15.

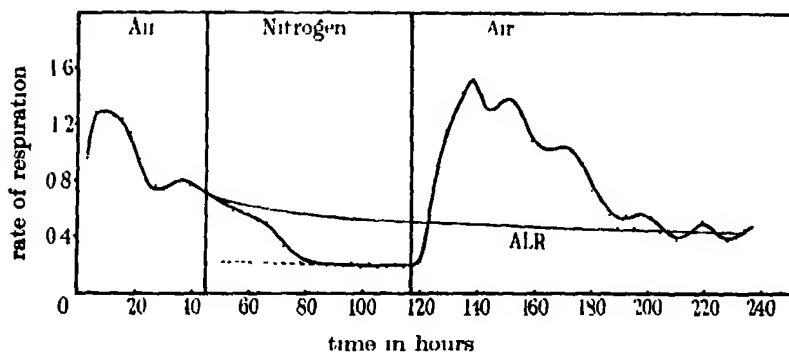


FIG. 5. Potato, Exp. 16.

In Exps. 15 and 16 (figs. 4, 5), the samples of potatoes were subjected to nitrogen for 72 hr. each, but in one case they were brought back to air and in the other to a lower concentration of 6.2% oxygen. The quantitative aspect of these two experiments (taking "air-line" as the standard) are set out in Tables I and II, together with three others where the anaerobic

period varied from 24 to 144 hr. In all cases there was a fall in carbon dioxide production in nitrogen, followed in air by a rapid rise to a value greater than that of the normal air-line, and then a gradual fall until the normal rate was resumed. Exp. 17 (Table II) was spoiled by fungal attack so it was not possible to determine the number of hours it would have taken to regain the "air-line" rate nor the total evolution of carbon dioxide during that period. From Table I as well as from figs. 4 and 5, it will be seen that the lowering in the rate of anaerobic carbon dioxide production, as compared with the rate indicated by the corresponding "air-line", proceeded in three stages, (1) a slow fall for the first few hours, which was followed by (2) a rapid fall. The third stage was reached after about 48 hr. in nitrogen after which time there was little change in the rate of carbon dioxide production. This steady rate, once attained, could be maintained even for 6 or 7 days without further alteration.

TABLE I. RATIOS OF NR TO ALR IN POTATOES

Hours in nitrogen	Exp. 15			Exp. 16			Exp. 17			Exp. 18			Exp. 19		
	NR			NR			NR			NR			NR		
	NR	ALR	ALR	NR	ALR	ALR	NR	ALR	ALR	NR	ALR	ALR	NR	ALR	ALR
12	0.52	0.54	0.96	0.59	0.64	0.92	0.51	0.58	0.88	0.64	0.62	1.03	0.57	0.60	0.95
24	0.38	0.49	0.775	0.45	0.59	0.763	0.34	0.53	0.64	0.46	0.56	0.82	0.40	0.56	0.714
36	0.26	0.46	0.565	0.22	0.56	0.39	0.23	0.50	0.46	0.30	0.53	0.566	—	—	—
48	0.22	0.43	0.51	0.20	0.53	0.38	0.20	0.19	0.41	0.26	0.52	0.500	—	—	—
60	0.20	0.43	0.465	0.20	0.50	0.400	0.20	0.48	0.416	—	—	—	—	—	—
72	0.20	0.42	0.476	0.19	0.50	0.38	0.20	0.47	0.425	—	—	—	—	—	—

TABLE II. QUANTITATIVE ASPECTS OF THE RESPIRATORY RATE  
OF POTATOES IN AIR AFTER NITROGEN TREATMENT

(All respiration rates are expressed as mg. carbon dioxide evolved per hour per 100 gm. fresh weight)

Exp.	Hours in nitrogen	Maximum respiration rate in air after nitrogen	Corro- sponding ALR	Max. R ALR	Time to reach maximum	Time to regain ALR	Total CO <sub>2</sub> in mg. evolved after retransference to air before the normal rate of respiration is resumed
15	72	0.72	0.42	1.71	24	115	62.32
16	72	1.52	0.48	3.17	21	90	86.30
17	144	1.62	0.48	3.37	30	—	—
18	48	1.30	0.50	2.6	15	93	83.43
19	24	0.67	0.55	1.22	15	27	15.63

An observation regarding the relation of lenticels and surface area to respiration may be worth putting on record here. Two experiments were

run concurrently, in one of which three potatoes formed a sample (Exp. 7) while in the other the number of potatoes was six (Exp. 8). The total fresh weight in the former case was 292 g. while in the latter it was 340 g. At the end of the experiments the number of lenticels and the area of the skin were determined (Michaels 1932).

In Exp. 7 the total area of the three potatoes was 32.95 sq. cm., with a total of 615 lenticels, that is, in the proportion of 18.66 lenticels per sq. cm. of the skin. The total amount of carbon dioxide produced in 120 hr. was 60.285 mg. In Exp. 8 the surface area was 44.59 sq. cm. with 903 lenticels, that is in the proportion of 20.25 per sq. cm. The total amount of carbon dioxide produced in 120 hr. was 69.780 mg. Although too much stress should not be laid on these few observations, it may be noted that although the smaller potatoes had more lenticels per unit area, this was not related to a greater output of carbon dioxide, since the respiration rate per unit weight of material was practically identical in the two cases.

## (2) Carrot

The normal course of the respiration of carrots in air is shown in fig. 6 (Exp. 21). The carrots were dipped in 0.2% mercuric chloride solution, but a control experiment run simultaneously did not show any significant difference in the intensity of respiration. This was also true for beetroots and artichokes. The carrots, however, tended to turn brown in patches at the end of an experiment, especially when they were bruised to start with.

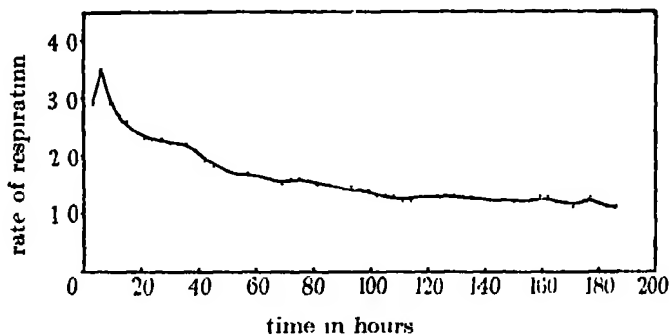


FIG. 6. Course of respiration of carrot root in air, Exp. 20.

The course of respiration of carrot roots in nitrogen is shown in fig. 7 (Exp. 27), the material in this case having been kept in nitrogen for 117 hr. after an initial period of 48 hr. in air. The quantitative aspects of this experiment and four other similar ones are shown in Table III. It will be



seen that in every case the rate of respiration in nitrogen exceeded that in air.

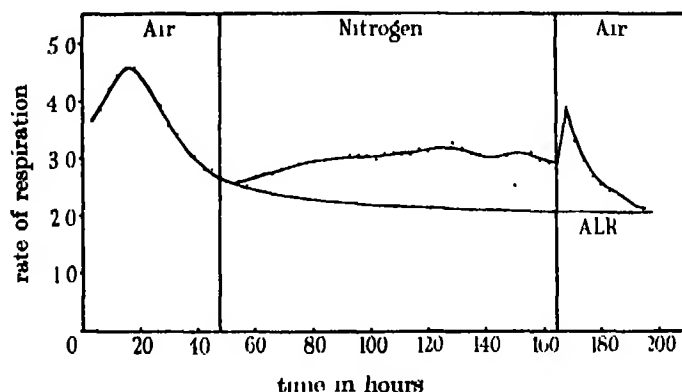


FIG. 7. Carrot, Exp. 27.

TABLE III. RATIOS OF NR TO ALR IN CARROTS

Exp.	Time in hours	NR/ALR
26	21	1.31
	51	1.42
27	24	1.21
	72	1.46
	96	1.45
	117	1.41
34	24	1.19
	48	1.51
	72	1.49
39	21	1.22
	48	1.23
40	51	1.48

The behaviour of carrot root in different concentrations of oxygen stands in marked contrast to that of potato tuber, for carrot root is very markedly affected by variation of oxygen concentration in the environment. This is shown in fig. 8 (Exp. 35) where the oxygen concentration ranged between 6.2 and 100 % and also in Table IV, in which are brought together all the experiments carried out with carrot in oxygen and artificial gas mixtures. Attention is drawn to Exps. 37, 38, 39 and 40 in which the tissue was exposed to a low (3.5 %) oxygen concentration; the results of two of these experiments are shown graphically in figs. 9 and 10. Unfortunately both the experiments illustrated here were spoiled by fungal attack towards the end of the experimental period, so the "air-lines" were not established beyond doubt, but those drawn in the figures are based on a comparison of analogous

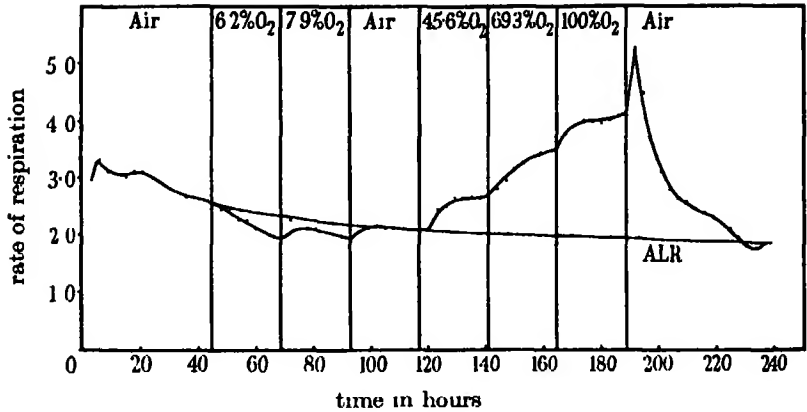


FIG. 8. Carrot, Exp. 35.

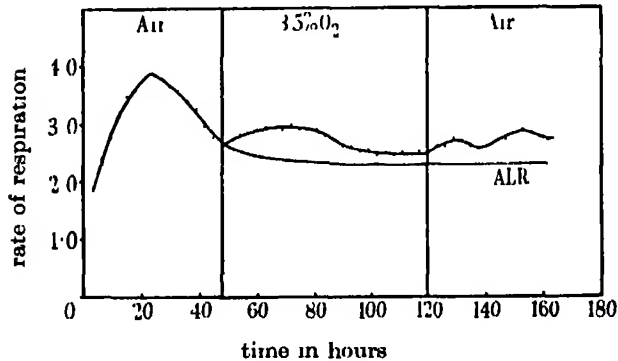


FIG. 9. Carrot, Exp. 37.

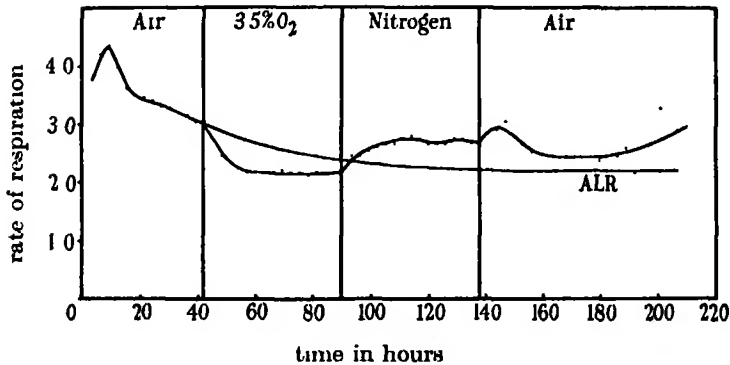


FIG. 10. Carrot, Exp. 39.

cases. In these two figures, 9 and 10, it will be seen that although in one case the respiration rate rose above and in the other case fell below the respective "air-lines", yet as exposure to low oxygen concentration continued there was a tendency for the respiratory rate to regain the "air-line" level, and perhaps in Exp. 37 (fig. 9) it was actually regained after about 48 hr. in 3.5 % oxygen. This was also the case with higher concentrations of oxygen, where the increased respiratory rate attained the maximum within a few hours, usually within 15 hr. (see Table IV), and then gradually began to decrease to approach the "air-line" level. Exp. 40 supplied some results intermediate between those of Exps. 37 and 39 (figs. 9 and 10). In this case for about 12 hr. the respiratory rate in 3.5 % oxygen was below the "air-line", after which time it began to rise and attained the maximum in 27 hr., this rate more or less being maintained for the rest of the period in 3.5 % oxygen (Table IV). It may be mentioned here that in these last four experiments (Exps. 37 to 40), the wounds in the carrots caused by the

TABLE IV. RATIO OF OR TO ALR IN CARROTS IN  
DIFFERENT OXYGEN CONCENTRATIONS

Exp.	Concentration of oxygen	Time in hours	OR/ALR
22	79.9	21	1.74
		45	1.51
23	79.9	21	1.66
		69	1.36
24	100	12	1.27
		96	1.11
25	100	15	1.26
		93	1.23
28	25.5	24	1.0
		72	1.0
29	24.4	12	1.15
		30	1.0
30	40	12	1.25
		96	1.13
31	7.9	36	0.91
		72	0.76
32	7.9	24	0.78
		69	0.75
35	6.2	24	0.67
		69	0.72
37	3.5	24	1.23
		72	1.09
39	3.5	9	0.803
		48	0.91
40	3.5	12	1.00
		27	1.12

removal of leaves were covered with "air-tight" grafting wax. The intensity of respiration in these cases, however, was not affected by this treatment.

### (3) *Red beetroot*

Only two experiments were carried out with roots of red beet. They were primarily undertaken to see if there was any similarity existing between the anaerobic respiratory rate of the roots of carrot and beet, since both of these materials are known to store a considerable amount of sugar.

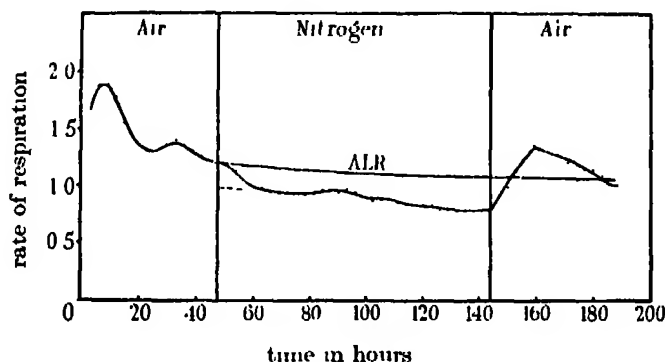


FIG. 11. Red beetroot, Exp. 42.

In the first experiment the course of respiration of beetroots in air was examined. It followed a fluctuating course which exhibited a slow decrease in respiratory activity with time. In the other experiment, which was run simultaneously with the previous one, the material was subjected to anaerobic conditions for 96 hr. after an initial period of 48 hr. in air. The results of this experiment are shown in fig. 11 while the quantitative aspects of the experiment are shown in Table V which also includes those with artichokes. At the end of this experiment the material was found to be infected by a fungus although it was quite turgid and in a good condition. The rise in respiration rate above the "air-line" in air after the anaerobic period might have been due, at least partly, to fungal activity.

### (4) *Artichoke*

The work with artichoke began late in the season, and after a time it became increasingly difficult to get suitable material. The results with this tissue are thus rather fragmentary but are of interest in comparison with the results obtained with other tissues.

The normal course of the respiration of artichokes in air is similar to that observed with other tissues, exhibiting an initial rise of short duration followed by a slow fall. In fig. 12 are shown the results of one of the three experiments carried out in air and nitrogen. The quantitative aspects of all of them are shown in Table V. In fig. 13 are shown the results of Exp. 47 in which the artichokes after a period in air were subjected to pure oxygen, then retransferred to air and then again to pure oxygen. Similar results

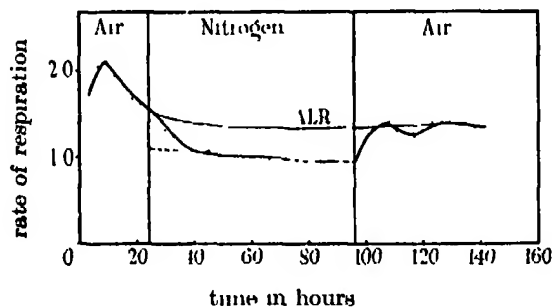


FIG. 12. Artichoko, Exp. 45.

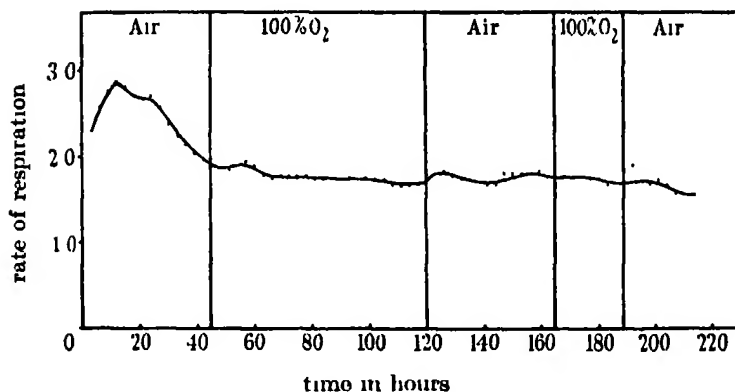


FIG. 13. Artichoko, Exp. 47.

were obtained in a second experiment with pure oxygen (Exp. 48) as well as in two experiments (49 and 50) with 51.6 % oxygen. In fig. 14 (Exp. 51) are shown the effects of a series of increases of oxygen concentration from 3.5 % to that in air. In air the respiratory rate actually rose beyond the "air-line" and maintained a higher level for a considerable time. A similar phenomenon was also observed in another experiment in which the tissue was subjected to a low concentration of oxygen (Exp. 49), where the respiratory rate of artichokes after a period of 75 hr. in 6.7 % oxygen rose,

on re-transference to air, to a maximum of 1.38 times that of the corresponding normal "air-line" value 27 hr. after this re-transference. This high rate, however, was followed by a fall to the normal rate in about the

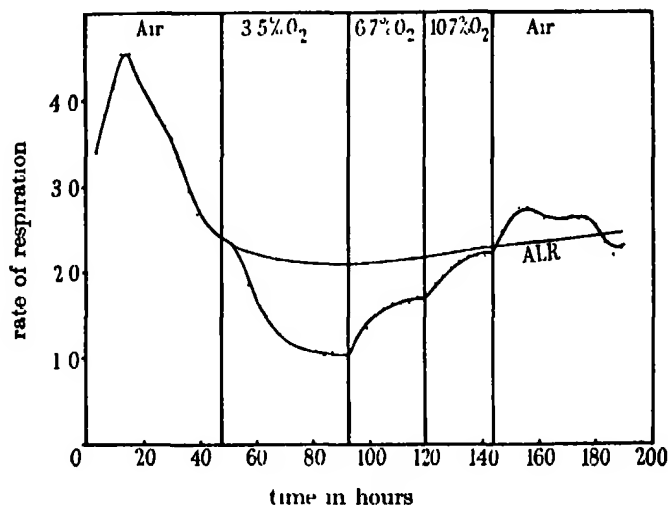


FIG. 14. Artichoke, Exp. 51.

TABLE V. RATIOS OF NR TO ALR IN RED  
BEETROOTS AND ARTICHOKE TUBERS

Tissue	Exp.	Time in hours	NR/ALR
Red beetroot	42	24	0.82
		48	0.81
		72	0.75
		96	0.72
Artichoke tuber	44	24	0.84
		48	0.76
	45	24	0.74
		72	0.73
"	46	24	0.75
		48	0.675
		72	0.62
		96	0.62
"	42	24	0.82
		48	0.81
		72	0.75
		96	0.72

next 21 hr. It will be seen that no such high rate of respiration in air was observed after an anaerobic period in nitrogen (fig. 12). The artichokes in Exp. 51 (fig. 14) were very advanced in age to start with and each already had one developing bud, which was removed. At the end of the experiment,

however, several buds had developed. In one experiment (no. 50) the course of respiration of the sprouting tubers was followed for over 80 hr. It was found that the respiratory rate tended to become irregular but on the whole increased slowly with time.

### DISCUSSION

From an examination of the experiments illustrated in the text it will be seen that the normal drift of the respiratory activity of all the materials under the experimental conditions went through a two-phase course, first a rise for a few hours to a maximum, and then a fall. The ultimate course of this slowly falling rate was, however, determined by the age, and perhaps the kind, of material used. Thus an analysis of more than twenty experiments carried out with potatoes showed that the respiratory rate of tubers used early in the storage season continued to fall, although very slowly, throughout the experimental period, while the respiratory rate of those used late in the season sooner or later assumed a level course. In the case of artichokes it was found that if the tubers were very advanced in age, there was a tendency for the respiratory rate to rise with the extension of the experimental period. In these cases as well as in some of the older potatoes, the tubers usually began to germinate towards the end of an experiment, and the increased rate of respiration might be due to the buds present on them. Nevertheless, a consideration of all the results obtained in this investigation suggests that there may be some seasonal drift in the respiratory rate of potatoes and possibly also in other cases. An examination of the data presented by fifty-one experiments suggests that the respiratory rate of potatoes at the beginning of a season is higher than that observed in the middle of the season. Later there is a tendency for the respiratory rate to increase as the season advances. This is probably associated with the normal function of propagation carried out by these organs.

The excess production of carbon dioxide at the beginning of an experiment is probably due to change of temperature. The previous history of the materials is not known, but if it is assumed that they were stored in a temperature of about 15 or 16° C, then under the experimental conditions they were heated up by about 9 or 10° C. In that case more than one factor may contribute to the excess carbon dioxide output (Blackman and Parija 1928). One of these factors must be the evolution of dissolved and easily dissociable carbon dioxide from the tissues, since the solubility of the gas would be lower at a higher temperature (Willaman and Brown 1930). Dissolved carbon dioxide can also be given out if the partial pres-

sure of this gas is lowered in the environment. This was observed every time work was resumed after an interruption. With the sudden change of temperature, the starch-sugar balance also might play a part, at least in potatoes, in the excess production of carbon dioxide (Blackman and Parija 1928; Singh and others 1937).

A comparison of the results recorded in the text indicates that the intensity of respiration in different materials is not the same; the average rates of carbon dioxide production at 100 hr. were 0.46, 2.00, 1.57 and 1.26 mg./100 g. fresh weight per hour in potatoes, carrots, artichokes and beetroots respectively. The average percentage losses in weight of the materials per 100 hr., which must have been largely due to the loss of carbon dioxide and water, were 0.51, 4.95, 2.65 and 2.04 respectively in potatoes, carrots, artichokes and beetroots. It is interesting to note the relation existing between the average production of carbon dioxide recorded above, and the average percentage loss in weight of the materials.

It will be seen that the respiratory behaviour of all the materials in presence of different oxygen concentrations was not similar. The increased respiratory rate of carrots with increase of oxygen concentration in the environment (fig. 8) may be due to an acceleration of the production rate of the immediate substrate for respiration. Thus Blackman (1931) said that: "A further survey of the facts produces evidence that oxygen has a dual effect upon the rate of respiration. One of these is the direct oxidative effect of oxygen upon a carbon substrate, while the other presents itself as an activation of the carbohydrate metabolism which determines the production rate of the specific substrate which is oxidized." If this statement is applicable in general, it is difficult to see why oxygen should not affect the production rate of the respiratory substrate in potatoes and artichokes. In the lowest concentration of oxygen used, 3.57 %, the respiratory rate was determined, as has been said already, by the size of the carrots; if a sample consisted of thin and small carrots, the respiratory rate was much below normal (Exps. 38 and 39, fig. 10), but if the carrots were massive the rate was above the normal (Exps. 37 and 40, fig. 9). In this latter case the excess carbon dioxide must have been derived from the anaerobic source. The size of the carrots will thus be a factor in determining the value of the "extinction point of NR". In Exp. 38 the "extinction point" lay between 3.5 and 6.7 % oxygen (Table III), but probably very close to the former percentage. There was no indication that this critical percentage of oxygen was more than 6.7 % in any of these experiments with carrots.

The respiratory rate of artichoke did not change from the normal when



the concentration of oxygen was increased from that of air to 51.6 or 100 % and vice versa. It was, however, reduced in lower concentration of oxygen (Exp. 51, fig. 14). If this is due to a shortage of oxygen for complete oxidation of sugar and if the production rate of the respirable substrate is not slowed down, there should be an accumulation of such substrate (or an anaerobic product of it, or both) which may readily be used up when sufficient oxygen is available. This in fact was realized in both cases (Exps. 49 and 51, fig. 14) when the artichokes were brought back to air from a lower concentration of oxygen.

The absence of any alteration from the normal course of the respiratory rate of potatoes under the influence of various oxygen concentrations may be due to the fact that even the lowest concentration used in these experiments, namely, 6.2 % oxygen, was sufficient for the complete oxidation of the respiratory substrate. This may not be impossible when the low intensity of respiration of these tubers is taken into consideration (cf. Exp. 15, fig. 4, where 6.2 % oxygen seemed to be enough as far as normal oxidation is concerned), yet one is naturally led to wonder what might be the amount of oxygen available at the centre of a tuber when the outside concentration is only 6.2 % and how there can be complete oxidation when the oxygen supply must be so limited. From these considerations (taking also into account the anaerobic respiratory rate in carrots) one thing seems clear, that even if oxygen can stimulate the supply of respirable sugar in certain cases it is not essential to it. In potatoes and artichokes, oxygen does not seem to play any part at all in the production of the respiratory sugar.

The rate of respiration of these storage organs in nitrogen was also different in different cases. In artichoke and beetroots there was a decrease in the respiratory rate which was rapid for the first few hours but slowed down considerably with time; in potatoes the decreasing rate took a much longer time to attain a level course which was maintained for the rest of the period in nitrogen. The ratios  $NR/ALR$  also were not the same in all the cases (Tables I, IV and V), and in carrots, in contrast to the other tissues examined, there was an increase in the respiratory rate under anaerobic conditions, rising steadily and gradually for more than 50 hr. before there was any tendency for a fall (Exp. 27, fig. 7), and even after 117 hr. in nitrogen, the anaerobic rate of carbon dioxide output was far above the normal rate (Exp. 27, Table III). When the carrots were brought back to air from nitrogen, there was always a rapid fall to the "air-line" level. Thus in these experiments the increased respiratory rate in nitrogen was not temporary but long maintained. In no case was there

observed any indication of a deterioration of the respiratory system in any of these materials as a result of nitrogen treatment.

There are cases on record in the literature where the anaerobic respiratory rate is one-third the aerobic, as, for example, in germinating *Fagopyrum* seeds (Leach 1936). In these cases the product of glycolysis in air is perhaps fully oxidized, but where this theoretical ratio is more than one-third, it is possible, if all the anaerobic carbon dioxide is derived from fermentation, either that the glycolysis rate, somehow or other, is increased in nitrogen (Dixon and Holmes 1935), or that a part of the product of glycolysis in air is built back into the system in the hypothesized process called oxidative anabolism (Blackman).

The data obtained for the anaerobic respiration of potatoes, artichokes and beetroots, afford an opportunity for estimating the oxidative anabolism (OA) that might take place in these materials. The ratios of initial NR (which are obtained by the method of Blackman 1928) to the final OR (respiration in air) are set out in Table VI. Only six experiments are considered here, because in them the anaerobic period was long enough for the NR rate to be established after a transitional period following the change from air to nitrogen. The results obtained with carrots, however, do not very readily allow one to find the initial NR rate by the above method and are therefore excluded from the table.

TABLE VI. RATIOS OF INITIAL NR TO OR  
IN VARIOUS TISSUES

Exp.	Material	NR/OR
15	Potato	0.36
16	"	0.306
17	"	0.34
42	Beetroot	0.81
45	Artichoke	0.70
46	"	0.72

If these values provide information of the glycolysis rate in air, it can be said that in potatoes there is little or no OA, while in artichokes and beetroots, a considerable amount of OA goes on. But in no case is the rate as high as that found in senescent fruits (Blackman 1928; Singh *et al.* 1937).

Attention may be drawn to the fact that the fall in the respiratory rate of potatoes, when air was replaced by nitrogen, to a constant lower level, usually took a long time. This time, indeed, seems too long to be explained as a diffusion lag, and can with more likelihood be ascribed to changes in

pre-glycolytic processes leading to a reduction in the concentration of the substrate for glycolysis and hence to a reduction in the rate of glycolysis. The maintenance of this minimum rate at a constant level for a considerable period may perhaps be explained by the presence of the large supply of substrate available for respiration in potato tubers.

Unlike other materials employed in these experiments, the respiratory rate of potatoes increased greatly when they were brought back to air (or even to as low as 6.2% oxygen, Exp. 15, fig. 4) from an atmosphere of nitrogen. Samples of potatoes have been subjected to nitrogen for varying lengths of time and their quantitative relations are shown in Table II. From this it will be clear that the ratio of the maximum OR (in air) value to that of the corresponding ALR increased with an increase in the duration of the anaerobic period, but was not always proportional (Singh and others 1937). The time required to regain the normal rate in air after an exposure to nitrogen and the total amount of carbon dioxide produced in that time hardly increased with an extension of the anaerobic period beyond 48 hr.

The increased respiratory rate of potatoes in air (or in 6.2% oxygen) after a period in nitrogen might be due to one or more causes. It may be that some oxidizable substance is produced during the anaerobic period and is used up when sufficient oxygen becomes available, or it may be that some substance is produced in nitrogen which afterwards acts merely as a stimulant and increases the aerobic respiratory rate (Meyerhof 1925). A third explanation can be that a temporary protoplasmic change of the type of Blackman's "lowering of the organization resistance" is brought about when nitrogen is replaced by air, which leads to an increased availability of sugar for oxidation. At present it is difficult to come to a definite conclusion as to the cause of this increased rate of respiration in potatoes, and until an investigation is made of the simultaneous chemical changes it is not advisable to carry the present discussion any further.

Last of all it may be mentioned that the cause of many of these differences observed in the respiratory behaviour of various plant materials may lie to a great extent in the specific nature of protoplasm, and it may be that until our knowledge of this substance is increased no very satisfactory explanation will be found for many of these observations.

The author takes this opportunity to express his gratitude to Professor W. Stiles for suggesting the problem and also for his stimulating interest and help.

## SUMMARY

Changes in the respiration intensities of the tubers of potato and artichoke, and the roots of carrot under a wide range of oxygen concentration, were measured continuously for several days at 25° C by the Pettenkofer method. The rate of aerobic and anaerobic carbon dioxide output by red beetroots was also measured.

The normal respiratory rate in air in all the materials usually went through a typical two-phase course, first a rise to a maximum, and then a slower fall which in some cases reached a constant level. The first rise may largely be due to the evolution of dissolved carbon dioxide from the tissues as a result of temperature change.

Indication of a seasonal drift of respiratory activity was observed both in potatoes and artichokes.

At 100 hr. the average carbon dioxide production was 0.46, 2.00, 1.57 and 1.26 mg./100 g./hr. in potatoes, carrots, artichokes and red beetroots respectively.

In potatoes the normal respiratory activity was maintained in different oxygen concentrations ranging between 6.2 and 98.6 %; in artichokes the respiratory rate altered with an alteration in the lower concentrations of oxygen, but when the oxygen concentration was above that in air there was no change from the normal course. In carrots the respiration rate rose with every rise in the oxygen concentration. In 3.5 % oxygen the size of the carrots determined the rate of carbon dioxide production.

In nitrogen the respiratory rate of potatoes, artichokes and red beetroots was reduced; in carrots the anaerobic rate gradually rose above the normal and maintained the increased rate even for 117 hr.

A change from anaerobic to aerobic conditions (or even to 6.2 % oxygen) was accompanied by a rapid rise in the respiration rate of potatoes, but the amount of carbon dioxide given out in air before the normal rate was resumed did not always show a direct relation with the length of the period of anaerobiosis.

From various considerations oxygen does not seem to be essential for the production of respiratory sugar, neither does it seem to have any direct control over the rate of production.

Smaller potatoes had more lenticels per unit area of the skin but that did not prove to be an advantage in carbon dioxide production.

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## The histology and self-differentiating capacity of the abnormal cartilage in a new lethal mutation in the rat (*Rattus norvegicus*)

BY HONOR B. FREIL, *Messel Research Fellow,  
 Strangeways Research Laboratory, Cambridge*  
 AND HANS GRÜNEBERG, *Moseley Research Student,  
 Department of Biometry, University College, London*

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[Plates 3–5]

### INTRODUCTION

In a previous communication (Grüneberg 1938), a new recessive lethal mutation has been described in the rat which produces a variety of anomalies in various parts of the body. It was shown that all these deviations from the normal, including those disturbances which lead to the death of the lethals, are ultimately caused by an anomaly of the cartilage. All the other manifestations of the gene are therefore of a secondary nature. The histology of the abnormal cartilage will be described in the first part of this paper.

For the anomaly of the cartilage, no obvious cause could be discovered by morphological means. It was pointed out, however, that this does not necessarily mean that the gene acts primarily on the cartilage. There remained the possibility that the cartilage itself was only secondarily affected by some general physiological condition of the body which produced no other visible changes.

For reasons discussed below, it is probably impossible to decide whether the gene acts directly or indirectly on the chondrogenic cells. By suitable methods, however, it can be ascertained whether there exists in the body of the lethals general conditions which cause the development of the cartilage anomaly and whose effect is *reversible*. If there existed, for instance, a chemical compound which caused the cartilage cells of the lethals to differentiate on abnormal lines, this compound should exercise a similar effect on normal cartilage implanted into the lethal animal. Similarly, if cartilage were removed from a lethal and grown in a normal environment, the hypothetical substance should gradually disappear from the cartilage as a result of diffusion or metabolism. At any rate it should be diluted as the growth of the transplanted cartilage proceeded, and we might therefore expect that the subsequent development of the transplant would be comparatively normal. The second part of this paper deals with experiments intended to elucidate these questions.

## TECHNIQUE

### *Histological*

The tissue was fixed in 2% acetic Zenker's solution. When fairly hard bone was associated with the cartilage, the material was fixed in the usual way, hardened by running it through the alcohols to absolute, then again hydrated and decalcified for 15–30 min. in formol-nitric acid.

Sections were stained with azan, haematoxylin and chromotrop or Wilder's silver method.

### *Tissue culture*

Cultures were grown either by the usual hanging-drop method on  $1\frac{1}{4}$  in. square cover-slips over  $3 \times 1\frac{1}{2}$  in. hollow-ground slides or by the watch-glass method (Fell and Robison 1929). The culture medium consisted of a mixture of equal parts of fowl plasma and saline extract of 10–11-day fowl embryos (mammalian embryonic skeletal tissue grows and differentiates very well in this heterologous medium).

### *Implantation*

The rats into which tissue was to be grafted were subjected to deep ether anaesthesia. The donor was killed and the graft was dissected under sterile conditions. Sterilized instruments were used for the operation, but otherwise the aseptic precautions were slight. The skin was not shaved at the site of the operation and only in the 14-day rats (see Table II) was the skin wiped with 70 % alcohol and then only with the object of making a parting in the fur.

A short, sharp Graafian knife was pushed under the skin and moved to and fro until a subcutaneous pocket had been formed. The graft was lifted in a fine pair of forceps and pushed well into the pocket, the opening to which was then sealed with a dab of collodion dissolved in equal parts of ether and alcohol. In the first experiment the grafts were placed beneath the skin of the shoulder, but later it was found more convenient to insert the graft under the skin of the thigh.

Very little bleeding occurred during or after the operation and none of the wounds became septic.

## PART I. HISTOLOGY OF THE ABNORMAL CARTILAGE IN LETHAL RATS

The abnormalities which characterize the cartilage of lethal rats do not appear to the same degree or at the same time in all parts of the cartilaginous skeleton. The general development of the abnormalities will be described first, after which representative cartilages throughout the body will be considered with reference to the special features which each displays.

The material was obtained from six lethal animals and five normal litter-mates ranging in age from less than 16 hr. to 19 days. Additional material was provided by the various controls used for the experiments described in Part II.

### *General development of the abnormalities*

The first visible change in the cartilage of the lethal is the formation of a delicate capsule, staining a deep blue with azan, around some of the peripheral chondroblasts. Simultaneously both the nucleus and cytoplasm of the enclosed cells become denser and more deeply staining. The capsules thicken and many of the chondroblasts become binucleate (fig. 1, Plate 3)

apparently as the result of incomplete mitotic division. Numerous mitotic figures occur throughout the abnormal cartilage.

The perichondrium which, as in the normal animal, consists at first of a fibrous membrane containing numerous fibroblast-like cells, may begin to show abnormalities at about the same time as the cartilage. The cells nearest the cartilage enlarge, become oval or rounded (fig. 1, Plate 3; cf. fig. 3) and multiply actively. This cellular enlargement and proliferation spreads towards the outer surface of the membrane, whilst the inner cells become transformed into typical young chondroblasts (fig. 2, Plate 3; cf. fig. 3) and begin to deposit cartilaginous matrix. The original intercellular fibres of the perichondrium are incorporated in the cartilage where they can be readily distinguished in preparations stained with Wilder's silver method; later they become diffuse and disappear. The newly formed cartilage matrix is lightly staining and not very dense, but soon deeply staining pericellular capsules develop, like those described above. The extent to which the perichondrium proliferates is extremely variable in different cartilages, as will be seen later, but in extreme cases a broad "cortex" of young cartilage is formed around the original tissue (fig. 6, Plate 4).

At first the "cortex" is quite sharply defined from the pre-existing cartilage but later the boundary is largely obliterated. In the final stages of development perichondrial proliferation rapidly declines and in some parts of the skeleton seems to be almost arrested.

The pericellular capsules continue to enlarge (fig. 5, Plate 3). When slices of fresh cartilage are examined in saline, these capsules somewhat resemble starch grains and show well-marked, concentric layers of deposit. They are very easily detached by cutting or other manipulation of the cartilage and lie freely in the saline where they can be minutely examined in an unfixed condition. The concentric structure is also well seen in sections stained by Wilder's silver method (fig. 4, Plate 3).

In the more advanced stages of the abnormality large areas of cartilage degenerate. The cells die while the capsules and intercapsular matrix gradually disintegrate into a lightly staining material permeated by a network of fine fibrils. Eventually these areas seem to liquefy, which may cause distortion in the general form of the cartilage (figs. 6, 8, Plate 4). In other areas, although the intercapsular matrix becomes very faintly staining as in the liquefying regions, the capsules and enclosed cells remain unaffected and appear startlingly conspicuous against the light background.

In regions of ossification the pericellular capsules appear first at the margin of the growing zone of flattened cells lying between the small-



celled epiphysial region and the hypertrophic cartilage of the diaphysis. The capsule formation gradually spreads into the interior of the zone of flattened cells, but the capsules are usually less dense than at the periphery. The flattened cells in the affected regions are less compressed than in the normal; they are not all encapsulated and in places proliferate to form large, irregular groups with at first very little intercellular material. As cellular hypertrophy spreads into the zone of flattened cells, the capsules when present partly or wholly disintegrate to allow for the expansion of the enclosed cell. Ossification is always less advanced in the lethal rats than in the normal litter-mates.

*Abnormalities in individual cartilages*

As stated in a previous communication (Gruneberg 1938) the death of the lethal rats is due to various consequences of a hyperplasia of the cartilage of the ribs and trachea. For this reason the changes which occur in these cartilages will be described first.

*Costal cartilage.* In the new-born rat the abnormalities characteristic of the lethal are more pronounced in the sternal ends of the costal cartilages than anywhere else in the body. Cross-sections of the costal cartilages of lethal rats less than 16 hr. old have the following structure (fig. 1, Plate 3 and fig. 7, Plate 4; cf. fig. 9). The interior, for about half the total diameter of the section, is fairly normal except for the presence of many binucleate cells. Around this area is a zone in which each cell is enclosed by a narrow but sharply defined capsule, the capsules being thicker the nearer they lie to the periphery. The perichondrium is greatly thickened although the width varies in different parts of the same cross-section, and is packed with large rounded cells showing every stage of transformation from fibroblast-like cells near the periphery to typical chondroblasts next the original cartilage. Mitotic figures abound both in the perichondrium and in the peripheral chondroblasts.

A short distance from the sternal ends of the cartilages the changes are much less advanced. The outer zone of encapsulated cells is narrower, the capsules themselves are thinner, and although the perichondrial cells have in places begun to enlarge, the perichondrium is not greatly thickened. Further dorsal still, the perichondrium is almost normal, the capsules in the outer zone are extremely thin and binucleate cells are rare.

The abnormality develops rapidly. By about the 6th day after birth the appearances described above near the sternal ends have spread almost to the dorsal end, whilst near the sternum where the condition is always more advanced, the pericellular capsules have greatly thickened and the peri-

chondrium has formed a broad but very irregular cortex of young cartilage in which typical capsules have begun to appear. The proliferation and chondrogenic activity of the perichondrium itself, however, has somewhat declined in this region.

By the 17th day (fig. 8, Plate 4; cf. fig. 10), i.e. an age which is reached only by a minority of the lethals, the pericellular capsules are enormously thick and dense and the boundary between the original cartilage and the "cortex" of new cartilage is no longer distinguishable. In some of the cartilages, near the sternal attachment, the interior for about one-fifth of the diameter, is hopelessly degenerate (fig. 8). The perichondrium is densely fibrous but still proliferates and chondrifies in places, forming irregular nodules and projections on the surface which gives the rib an extremely irregular outline. The nodules of young cartilage formed at this stage differ from the earlier "cortical" cartilage in having smaller cells and a dense, fibrous matrix which gives a much fainter metachromatic staining with thionin than the adjacent original cartilage and a much darker coloration with azan. In places its appearance almost suggests osteoid tissue rather than cartilage. The ribs of normal litter-mates present a striking contrast to those of the lethal animals. In cross-section they are much smaller and have a regular, oval outline; the cells, which are arranged in groups, have no capsules but are embedded in fairly dense, evenly staining matrix.

*Trachea.* In the 16-hr. rat, the tracheal cartilage is almost normal although careful inspection shows that a very delicate, darkly stained capsule has been formed around some of the more peripheral cells.

By the 6th day the tracheal rings have become considerably thicker than normal, the pericellular capsules are more distinct and binucleate chondroblasts are common. In a few restricted regions the perichondrium has formed small lumps of new cartilage, usually on the edges of the rings, but it never shows much activity and nothing like the "cortex" of the costal cartilages is ever produced.

The abnormality continues to increase and by the 17th day (fig. 5, Plate 3) the capsules have become enormously thick and dense whilst the intercapsular material stains very lightly and seems to be disintegrating. The cartilage rings are now greatly swollen and distorted and far thicker than those of the normal animal at this stage.

*Larynx.* Although the larynx is so closely associated with the trachea, the cartilage does not become nearly so abnormal as that of the tracheal rings. In the 17-day animal the pericellular capsules are still quite thin and in places the cartilage is almost unaffected.

*Nasal septum.* At birth the nasal septum of the lethals is indistinguish-

able histologically from that of normal litter-mates, but the characteristic pericellular capsules have appeared round some of the chondroblasts by the 4th day. As in the trachea, perichondrial activity is extremely slight although it occurs in a few limited areas.

The abnormality never becomes very advanced in the nasal septum. Nearly all the chondroblasts become encapsulated, but even in a 17-day lethal the capsules are thin as compared with those of the ribs and trachea, whilst the intercapsular matrix appears quite normal.

*Ear.* No abnormality could be distinguished in the ear cartilage. In the 17-day lethal rat, all the chondroblasts are enclosed by a thin capsule, but precisely similar capsules occur in the ears of normal litter-mates. Large numbers of binucleate chondroblasts are also present in both the normal and lethal ears.

*Vertebrae.* No abnormalities could be detected in the sacral vertebrae of a 16-hr. lethal. In the 4-day rat changes are seen in the lumbar vertebrae (the sacral vertebrae were not examined). The perichondrium of the unossified cartilage of the centra has begun to proliferate slightly and has formed an incomplete layer of new cartilage on the ventral surface of each centrum. Pericellular capsules have also appeared and are thickest and most dense in the fibro-cartilage of the intervertebral disks.

By the 17th day most of the cells in the non-ossifying part of the centrum are surrounded by comparatively thin capsules. Similar changes occur, although less conspicuously, in other parts of the vertebra, the only region apparently unaffected being the neural spine. Very little new "cortical" cartilage is formed anywhere in the vertebrae.

It is a curious fact that no histological abnormalities could be detected in the vertebrae forming the tip of the tail even in a 17-day lethal.

*Sternum.* The sternum of the lethal animals is seriously affected and shows more pronounced perichondrial proliferation than any other cartilage examined.

In the new-born animal slight changes are just visible and delicate capsules enclose many of the peripheral chondroblasts. The perichondrium soon begins to grow very actively especially in the xiphoid process. Thus by the 4th day a broad, regular "cortex", almost as thick as the original cartilage, has been laid down on both the dorsal and ventral surfaces of the xiphoid. All the cells of the original cartilage, but not those of the "cortex", are now encapsulated.

In the 12-day lethal the pericellular capsules have become much thicker throughout the sternum, although they are not as thick as those of the trachea and ribs. Large tracts of degeneration, like those described above,

have appeared between the "cortex" and the original cartilage in the xiphoid (fig. 6, Plate 4), which has become much distorted in consequence (for an illustration see Grüneberg 1938, fig. 3, p. 130).

*Limbs.* The skeleton of the limbs never becomes very abnormal. In the new-born animal, in some parts of the skeleton, a few delicate pericellular capsules occur in the zones of flattened cells immediately within the perichondrium. By the 17th day a layer of encapsulated cells has also appeared just beneath the articular surfaces of many of the epiphyses, whilst the capsules in the zones of flattened cells are thicker and more numerous. Broad, well-developed capsules, however, are only formed in the fibro-cartilage at the insertion of the tendons. In the interior of the cartilage, large tracts remain almost or completely normal throughout the life of the animal.

*Pelvis.* The pelvis shows the lethal abnormalities rather more strikingly than the skeleton of the limbs but much less so than the ribs and trachea. At birth the pelvis appears normal except for the presence of a few very fine pericellular capsules near the surface. By the 17th day, however, nearly all the cells in the zones of flattened cells adjacent to the ossification centres and many of those near the articular surface of the acetabulum and in the anterior end of the ilia have acquired fairly thick, well-marked capsules. In some places also the perichondrium is proliferating slightly to form small masses of new cartilage.

## PART II. SELF-DIFFERENTIATING CAPACITY OF THE CARTILAGE ABNORMALITY OF THE LETHALS

### *Behaviour of abnormal cartilage in vitro*

*Object of experiments.* To find whether lethal cartilage would continue its abnormal development when isolated *in vitro*.

If the abnormality were self-differentiating it might be expected to progress when the cartilage was cultivated *in vitro*, since normal (embryonic) skeletal tissue grows and differentiates readily under these conditions.

*Material and methods.* Watch-glass cultures were made of costal cartilages from a 4-, 5-, 7-, 10- and 12-day lethal and also from a normal litter-mate of each. The rib cartilages from one side of each rat, after being separated from each other and the surrounding muscle, were explanted, whilst those of the opposite side were fixed and sectioned as controls. Thirty-five explants of lethal and nineteen explants of normal cartilage were grown

for periods ranging from 4 to 17 days and were then fixed and serially sectioned.

Hanging-drop cultures were made from one 13-day lethal and one normal litter-mate. In either animal three explants were taken from each of four rib cartilages, the cartilages from the opposite side being fixed and sectioned. The cultures were fixed after 13 days' growth.

*Results.* The abnormality progressed very little, if at all, during cultivation.

Camera-lucida drawings made at intervals during the culture period showed that growth of the cartilage was very slight or absent, although there was a varying amount of outgrowth from the surrounding soft tissue. In sections it was seen that proliferation and chondrogenesis in the perichondrium stopped soon after explantation so that the cartilage became sharply defined from the fibrous perichondrium as in the normal rib. In some of the explants the pericellular capsules seemed a little thicker and more numerous than in the controls, but in others the capsules were rather thinner, owing to the fact that the cells had enlarged during cultivation, and in the process of expansion had resorbed the adjacent layers of matrix. With a few exceptions the explants remained healthy after 12-14 days *in vitro*.

The explants of normal costal cartilage also survived unchanged and showed little growth.

*Conclusions.* The results admit of two possible interpretations: (a) the characteristic abnormality of the lethal cartilage is not self-differentiating but is caused by general physiological conditions in the animal, or (b) the abnormality is self-differentiating, but the conditions of tissue culture are not favourable for its development.

#### *Behaviour of normal cartilage when grafted into a lethal rat*

*Object of experiments.* To find whether normal cartilage would assume the abnormality typical of the lethal if it were grafted into a lethal animal.

If the first alternative mentioned above were correct, viz. that the abnormality characterizing the lethal cartilage was caused by the general physiological conditions in the animal, then normal cartilage subjected to the same physiological conditions should also develop the abnormality.

*Material and methods* (see Table 1). The costal cartilages were obtained from three normal foetuses, one near term and the others 3.5 cm. in length, and from three 3-day and three 2-day post-embryonic rats. The cartilages from one side were fixed and sectioned and those of the other were implanted separately into either a lethal rat or its normal litter-mate. The

grafts were maintained for periods ranging from 5 to 13 days and the host was then killed and the graft removed.

TABLE I. NORMAL COSTAL CARTILAGES GRAFTED INTO  
LETHAL RATS

Donors	Graft no.	Host		Duration of im-plantation days	Comments
3-day rat (litter-mate to hosts)	1	3-day lethal	} litter-mates	—	Rat eaten by foster-mother
	2	"		13	
	3	3-day normal		13	
2-day rat (unrelated to hosts)	1	5-day lethal	} litter-mates	—	Died
	2	"		8	
	3	"		8	
Foetus near term (unrelated to hosts)	1	12-day lethal	} litter-mates	5	Graft disappeared (bad im-plantation)
	2	"		7	
	3	12-day normal		5	
	4	"		7	
	5	5-day lethal	} litter-mates	10	
	6	"		10	
	7	5-day normal		—	
	8	"		—	
	9	4-day lethal	} litter-mates	—	
	10	"		—	
	11	4-day normal		10	
	12	"		10	
Two 3-5 cm. foetuses (unrelated to hosts)	1	7-day lethal	} litter-mates	9	Rat eaten by foster-mother
	2	"		9	
	3	"		9	
	4	"		—	
	5	7-day normal		9	
	6	"		9	
	7	"		9	
	8	"		9	
	9	5-day lethal	} litter-mates	11	
	10	"		11	
	11	5-day normal		11	
	12	"		11	

*Result.* The normal histological development of the costal cartilages was unaffected by implantation into lethal animals.

*Results.* The implanted cartilage elongated considerably but remained normal (fig. 11, Plate 5).

Careful comparison of costal cartilages from the same animals, grafted

into lethal and normal rats respectively, showed no significant difference between the two series. Neither exhibited abnormal proliferation of the perichondrium or the formation of pericellular capsules.

*Conclusions.* (1) The characteristic abnormality of the lethal cartilage is not caused by the general physiological condition of the animal.

(2) The failure of the abnormality to progress *in vitro* was therefore probably due to the conditions of tissue culture being unfavourable for its development.

*Behaviour of abnormal cartilage when grafted into a normal rat*

*Object of experiments.* To find whether the abnormality characteristic of the lethals would progress when cartilage from a lethal was grafted into a normal rat.

If the abnormality were intrinsic in the lethal cartilage, it might require for its development environmental conditions which would encourage growth. This seemed probable in view of the abnormally active cellular proliferation which occurs in the lethal cartilage in its normal position in the animal. As shown above, such conditions are provided by the method of subcutaneous implantation but not by that of tissue culture.

*Materials and methods* (see Table II). Various cartilages were removed from a 6-day (exp. 1) and a 3-day (exp. 2) lethal rat and subcutaneously implanted into a normal animal. As a control, either part of the cartilage to be grafted or the corresponding cartilage from the opposite side was fixed and sectioned. In exp. 1 all the hosts were normal litter-mates of the donors and received only one graft apiece. In exp. 2 the host into which the pelvis graft was implanted was a litter-mate of the donor, but the other grafts were placed in 14-day rats which were unrelated to the donors. The 14-day animals each received two grafts: one of cartilage from the 3 day lethal and the other of the corresponding cartilage from a normal litter-mate of the lethal. Better results were obtained when the hosts were litter-mates of the donors, possibly because the animals were younger. The implants were maintained for periods of 5-50 days, after which the hosts were killed and the grafts removed for histological study.

*Results.* All the grafts grew vigorously and in all the characteristic lethal abnormality advanced markedly. The abnormality developed more slowly in the implants than in the lethal animal, probably owing to the fact that implanted tissue never grows quite so readily as the same tissue in its normal position in the body. On the other hand, the grafts could be maintained for a much longer period than the short life of the lethal rat, and

TABLE II. LETHAL CARTILAGE GRAFTED INTO NORMAL RATS

Donors	Graft	No.	Host	Duration of im-plantation days
Two 6-day lethals (litter-mates)	Costal cartilage	1	7-day normal (unrelated to donor)	5
	"	2	"	8
	"	3	"	8
	"	4	"	14
	"	5	"	21
	$\frac{1}{2}$ xiphoid		6-day normal (litter-mate to donor)	8
	$\frac{1}{2}$ one acetabular region		"	14
	$\frac{1}{2}$ trachea		7-day normal (unrelated to donor)	21
	Metatarsal	1	6-day normal (litter-mate to donor)	5
		2	"	8
One 3-day lethal and one 3-day normal litter-mate		3	"	14
		4*	"	—
	Costal cartilage	1 L }	Both in 14-day normal (unrelated to donor)	7
	"	1 N }		
	"	2 L }	"	14
	"	2 N }		
	"	3 L }	"	50
	"	3 N }		
	"	4 L }	"	50
	"	4 N }		
	$\frac{1}{2}$ xiphoid	L }	Both in 14-day normal (unrelated to donor)	14
		N }		
	$\frac{1}{2}$ one acetabular region	L	3-day normal (litter-mate to donor)	50
	"	N†	"	—
	Metatarsal	1 L }	Both in 14-day normal (unrelated to donor)	7
		1 N }		
	"	2 L }	"	14
	"	2 N }		
	"	3 L }	"	50
	"	3 N }		
	"	4 L }†	"	—
		4 N }		
	Proximal tarsals	L }	"	50
		N }		

*Result.* The lethal cartilage grafted into normal rats continued to develop their characteristic, atypical histology.

\* Rat eaten by foster-mother.

† Rat died under anaesthetic.



consequently it was possible to obtain the abnormality in a much more advanced form in the older implants than is ever seen in the lethal animal itself.

The behaviour of each type of graft will be described separately.

*Costal cartilage.* At the time of explantation the controls showed that in the 3-day rib cartilages (fig. 12, Plate 5) the abnormality was similar to, but slightly less advanced than, that in the 4-day ribs described in the first part of this communication, whilst in the 6-day cartilages it was at the same stage as that previously described in ribs of this age.

In the 5-8-day implants from the 6-day donor the perichondrium was proliferating vigorously and had formed large masses of "cortical" cartilage containing many mitotic figures. The perichondrium was growing in a much more diffuse way than in the normally situated lethal rib and the new chondrogenic areas seemed to be penetrating and invading the surrounding tissue. Proliferation, though much reduced, was still in progress in the 14- and 21-day grafts. The implants from the 3-day lethal showed much less perichondrial proliferation than those from the 6-day animal, possibly because they had been grafted into older rats. Near the ends of the cartilage, however, the perichondrium was growing and forming new cartilage fairly actively. In both series of grafts new tissue was sometimes produced which resembled osteoid rather than chondroid tissue (fig. 14, Plate 5) and in some cases unmistakable coarse-fibred bone had differentiated although the adjacent cartilage was not of the hypertrophic type characteristic of an ossification centre. Perichondrial proliferation had stopped completely in the oldest (50-day) grafts.

Enlargement of the pericellular capsules continued in both series of implants. In the two 50-day grafts (fig. 13, Plate 5) they were relatively enormous and very dense. The central area of degeneration seen in the normally situated lethal rib, increased in the implants and in the 50-day grafts had a very remarkable appearance. In azan preparations it was almost colourless except for an irregular felt-work of darkly stained fibres, was very wide and had a sharp outline so that the rib was reduced for a large part of its length to a thick-walled tube.

The normal costal cartilages grafted into the same animals as the 3-day lethal ribs elongated greatly but developed none of the abnormalities exhibited by the implants of lethal cartilage (figs. 15 and 16, Plate 5; cf. fig. 13).

*Trachea.* The trachea of the 6-day lethal donor was split longitudinally. One half was implanted into a normal litter-mate of the donor and the other was fixed and sectioned as a control.

In the control half, pericellular capsules which, though distinct were not yet very thick, had formed round most of the peripheral chondroblasts, but the interior of the cartilage was comparatively normal.

The graft was fixed after 21 days' growth when it presented a great contrast to the control. It had enlarged very much, nearly all the cells were surrounded by capsules which were much broader than in the controls, and some perichondrial proliferation was taking place. Near one end of the graft, apparently typical bone and osteoid tissue had developed on the surface of the cartilage.

*Xiphoid process.* Two grafts of the xiphoid process of the sternum were made, one from the 6-day and the other from the 3-day lethal. Each xiphoid was divided longitudinally, one half being fixed and sectioned as a control and the other half implanted.

The xiphoid process from a normal litter-mate of the 3-day lethal donor was treated in a similar way, half being implanted into the same animal as the 3-day lethal xiphoid and the other half being fixed.

In both lethal controls nearly all the cells of the original cartilage were encapsulated but the capsules, especially in the 3-day specimen, were comparatively thin. The proximal part of the xiphoid was enclosed by a broad cortex of new cartilage, which was much less differentiated in the 3-day than in the 6-day xiphoid.

Both grafts were much larger than the controls. The 6-day xiphoid which was fixed after 8 days' growth, showed very active formation of cortical cartilage throughout most of its length. A few chondrogenic areas were also present in the perichondrium of the 3-day xiphoid which had been fixed after 14 days' growth. The pericellular capsules had greatly increased in size and density in both implants and in the 14-day graft the two characteristic zones of degeneration had appeared between the original and the new cortical cartilage, as already described in the normally situated lethal xiphoid.

The implant of the normal half xiphoid had also grown much larger but showed no trace of the lethal abnormalities.

*Tarsals.* The proximal tarsals of the 3-day lethal were implanted and maintained for 50 days. A similar graft obtained from a normal litter-mate was implanted in the same host and fixed at the same time. The opposite foot from each animal was fixed as a control.

In the lethal control, a few very fine pericellular capsules were present in places immediately beneath the perichondrium, but otherwise the cartilage appeared normal.

Both grafts had grown very much and were largely ossified, although

extensive tracts of unossified cartilage remained. The graft of the lethal tarsals showed very advanced changes. Thick, dense pericellular capsules had been formed throughout the cartilage. In the normal graft pericellular capsules occurred only in regions of endochondral ossification where a layer of osteoid tissue had been deposited inside the cartilage capsules adjacent to the erosion surface. In the lethal tarsals, the orientation of the hypertrophic cells was chaotic, the cells being distributed in irregular groups or scattered singly, whilst in the normal graft they displayed the usual regular, columnar arrangement. In many places the lethal implant had acquired a broad zone of young cortical cartilage which was absent in the normal graft.

*Metatarsals.* Four metatarsals from the 6-day and four from the 3-day lethal were implanted and maintained for periods ranging from 5 to 50 days. Four metatarsals were also removed from a normal litter-mate of the 3-day lethal and grafted into the same animals as the lethal cartilages. The opposite foot of all three donors was fixed as a control.

In the 6-day lethal control metatarsals a few fine pericellular capsules occurred in the zones of flattened cells immediately beneath the perichondrium but the cartilage was otherwise normal. Pericellular capsules were also present in the 3-day lethal controls, but were so delicate that they could only be detected with a high magnification.

Capsule-formation continued in the grafts of the lethal cartilage and in the older implants had spread throughout the small-celled and flattened cartilage. In the grafts of the normal metatarsals, capsules occurred in the oldest (50-day) graft only; these were mostly osteoid deposits of the type described above, and were restricted to regions of endochondral ossification. As in the implanted tarsals, the flattened cells of the lethal metatarsals displayed a chaotic arrangement unlike the orderly columns seen in the normal grafts. In the lethal implants a little new cartilage was formed at the surface by the perichondrium.

Ossification had advanced considerably in both lethal and normal grafts. In one of the lethal implants the periosteum of the shaft had proliferated very actively and formed a relatively large swelling consisting of a mixture of secondary cartilage, osteoid tissue and bone.

It was noteworthy that even after 50 days' implantation the lethal metatarsals did not show the characteristic abnormality in nearly so marked a way as did the costal cartilages.

*Pelvis.* Perhaps the most striking example of self-differentiation of the lethal abnormality was provided by two grafts of part of the acetabular region of the pelvis.

One graft was taken from the 6-day lethal and was maintained for 14 days, and the other, from the 3-day lethal, was fixed after 50 days.

Part of each acetabular region was fixed as a control. In the 6-day control thin but distinct capsules were already conspicuous near the periphery and perichondrial proliferation had begun. Only a few very delicate capsules were present in the 3-day control and there was no perichondrial proliferation.

In the 14-day graft from the 6-day lethal, stout capsules enclosed most of the chondroblasts and the actively growing perichondrium had in places formed a broad cortex of very early cartilage similar to the cortex in the normally situated lethal xiphoid process.

The changes in the 50-day graft taken from the 3-day lethal were still more striking, especially in view of the fact that the cartilage was almost normal when implanted. A very broad cortex of new cartilage, the cells of which were heavily encapsulated, had been formed on the articular surface of the acetabulum. In places perichondrial proliferation was still in progress and nodules of very young cartilage were seen. The chondroblasts in the zone of flattened cells were arranged in irregular groups sometimes of considerable size, and most of them were not encapsulated. Elsewhere almost all the chondroblasts were enclosed by broad, dense capsules.

*Conclusion.* The abnormality characteristic of the cartilage of the lethal rat is intrinsic in the tissue, at least in post-embryonic life, and its further development does not depend on general physiological conditions peculiar to the lethal animal.

## DISCUSSION

The histological features of the cartilage of the lethals, viz. abnormal cell proliferation and the formation of dense pericellular capsules, suggest that the condition represents a hyper-activity of the chondroblasts. This is supported by the fact that when the physiological activity of the tissues was depressed, as in the tissue-culture experiments which almost completely inhibited growth, the development of the abnormalities was arrested in spite of the fact that the tissue remained healthy. The exact nature of the abnormality is at present completely obscure, but biochemical experiments have been planned in collaboration with the School of Biochemistry, Cambridge, which it is hoped may shed light on this problem.

The distribution of the abnormal cartilage is peculiar. Whereas ribs and trachea are greatly affected, minor anomalies only are found in the larynx and the nasal septum. Elsewhere in the skeleton only certain restricted

areas develop capsulated cells. It seems, however, as if every cartilage cell of the lethals has the potentiality for capsule formation. Thus the transplanted cartilage which lived in a normal host for 50 days developed capsules in regions which ordinarily, during the short life-span of a lethal, remain almost, if not quite, unaffected.

The distribution of the anomalous areas cannot be accounted for by the assumption of a critical time in development, such that cartilage formed before that critical stage remains normal, whereas cartilage formed later develops capsules. It has been shown previously (Gruneberg 1938) that in the ribs, at any rate, the anomaly is recognizable at birth. But by no means all the cartilage formed after birth is abnormal.

The transplantation experiments show conclusively that both normal and abnormal cartilage cells differentiate according to their origin, and independently of the general physiological conditions of the host. In the case of the lethals, this applies to cells which, at the time of transplantation, did not yet show any capsules. It is therefore safe to conclude that the capsule formation by the cells is not due to a reversible influence which acts during or shortly before the actual development of the anomaly.

The fate of the cartilage cells is therefore irreversibly determined beforehand. This situation allows of two possible interpretations. The cartilage cells are either determined directly by the genes which they carry, or else indirectly by some general physiological conditions of the body which acted irreversibly prior to transplantation. Transplantation experiments in general can only show that from the time of transplantation onwards, the development was autonomous or self-differentiating; they cannot exclude the possibility of dependent determination earlier in development. We are on the whole inclined to regard direct gene action on the cartilage as more likely in our case. This assumption could be strengthened, though not conclusively proved, by tracing the self differentiating powers of the cartilage further back in development. Such experiments will be attempted.

The expenses of this investigation were partly defrayed by the Medical Research Council to whom we should like to express our thanks. We are also indebted to Mr V. C. Norfield by whom the photomicrographs were taken.

#### SUMMARY

1. The two chief abnormalities which characterize the cartilage of the lethal rats are (1) the formation of thick capsules around the chondroblasts, and (2) very active proliferation and chondrogenesis in the perichondrium.

2. In rats of less than 16 hr. these abnormalities have only just begun to appear.

3. The abnormalities do not develop to the same degree or at the same time in all parts of the cartilaginous skeleton.

4. When cartilage from young lethal rats is explanted *in vitro* the cartilage survives in a healthy condition for about 12 days, but does not grow, and the abnormality progresses little if at all during cultivation.

5. Normal costal cartilage when implanted subcutaneously in young lethal rats grows well but remains normal.

6. Cartilage from young lethal rats when implanted subcutaneously into normal rats grows vigorously and continues to develop its characteristic abnormality. Given sufficient time, the transplanted cartilage develops the anomalies to an extent never reached during the short life-span of a lethal.

#### CONCLUSIONS

1. The general physiological condition of the post-embryonic lethal rat is not responsible for the abnormal development of the cartilage.

2. The abnormality characteristic of lethal cartilage is self-differentiating, at least in post-embryonic life.

3. Whether in early development the gene acts directly or indirectly on the chondrogenic cells cannot be determined from the present experiments which refer to post-embryonic material only.

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#### APPENDIX

It was pointed out previously (Grüneberg 1938) that the lethals suffer from an emphysema of the lungs which impedes the pulmonary circulation. To counter these difficulties, the lethals develop a muscular hypertrophy of the right side of the heart. Another compensating mechanism familiar in human emphysema has in the meantime been found in the lethals. As the respiratory surface of the lungs is reduced, the haemoglobin content of the blood, as measured by Gower-Haldane's method, and the number of erythrocytes increases. The accompanying table shows that in the first

litter no significant difference exists between the lethals and their normal litter-mates. Later the values for the lethals lie consistently above those of the normals. This is yet another example of the fact that some of the secondary gene effects in spurious pleiotropism are due to readjustments of the body. The organism deals with the pathological situation created by a mutant gene in precisely the same manner as if that disturbance had been caused by an extrinsic agent.

*The haemoglobin content (Gower-Haldane) and number of erythrocytes (millions/mm.<sup>3</sup>) in the blood of lethals (L) and their normal litter-mates (N)*

Litter	Age in days		Haemoglobin	Erythrocytes
78a	6	N	52	2.29
		N	58	3.55
		N	58	3.47
		L	55	3.59
		L	58	3.34
27l	6	N	64	3.77
		L	78	4.33
82a	7	N	62	3.32
		L	71	3.77
68b	11	N	63	3.87
		N	63	4.20
		L	81	4.72
76b	12	N	62	3.75
		N	62	3.67
		N	63	3.87
		L	78	4.60
		L	81	4.76
		L	85	5.46
6h	15	N	60	—
		N	60	—
		N	65	—
		N	65	—
		N	66	—
		L	94	—

#### DESCRIPTION OF PLATES

##### Abbreviations

<i>bi.c.</i>	binucleate cell.	<i>d.a.</i>	degenerate area.
<i>c.</i>	capsule.	<i>mi.</i>	mitosis.
<i>co.</i>	cortex.	<i>p.c.</i>	perichondrial cell.

##### Plate 3

FIG. 1. Transverse section of a costal cartilage from a lethal rat under 16 hr. old. Thin, darkly staining capsules have been formed round the chondroblasts some of which are binucleate. The cells of the perichondrium have begun to enlarge. (Azan;  $\times 675$ .)

FIG. 2. Transverse section of a costal cartilage from a 3-day lethal rat. The perichondrial cells are proliferating actively. (Azan;  $\times 675$ .)

FIG. 3. Transverse section of a costal cartilage from a normal 3-day rat, litter-mate of the lethal from which the rib shown in fig. 2 was taken. Note the compact, fibrous perichondrium, cf. fig. 2. (Azan;  $\times 675$ .)

FIG. 4. Longitudinal section of the sternum of a 17-day lethal rat showing a single binucleate chondroblast and its capsule. Note the laminated structure of the capsule. (Wilder; carmalum; light green;  $\times 1630$ .)

FIG. 5. Longitudinal section of the trachea of a 17-day lethal rat, showing the large capsules several of which contain binucleate cells. (Azan;  $\times 675$ .)

#### Plate 4

FIG. 6. Longitudinal section of the xiphoid process of a 12-day lethal rat. A broad "cortex" of new cartilage has been formed on the upper and lower surfaces of the process. Note the two degenerate areas immediately below the cortex. (Azan;  $\times 90$ .)

FIG. 7. Transverse section of the costal cartilage of the 5th sternal rib from a lethal rat under 16 hr. old. (Azan;  $\times 45$ .)

FIG. 8. Transverse section of the costal cartilage of the 5th sternal rib from a 17-day lethal rat. Note the heavily encapsulated cells and the central area of degeneration. (Azan;  $\times 45$ .)

FIG. 9. Transverse section of the costal cartilage of the 5th sternal rib from a normal rat under 16 hr. old, litter-mate to the lethal from which the rib shown in fig. 7 was taken. (Azan;  $\times 45$ .)

FIG. 10. Section of the costal cartilage of the 5th sternal rib from a 17-day normal rat, litter-mate to the lethal from which the rib shown in fig. 8 was taken. N.B. Owing to the fact that the posterior ribs of the normal animal are set at an angle to the sternum, this section is slightly oblique, whilst the section shown in fig. 8 is transverse as the posterior ribs of the older lethals are almost vertical to the sternum. The relative difference in size between the normal and lethal ribs is therefore considerably greater than appears in the two photographs. (Azan;  $\times 45$ .)

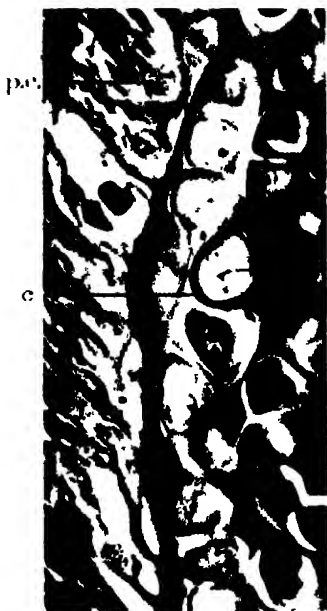
#### Plate 5

FIG. 11. Transverse section of a costal cartilage from a normal embryo near term, which had been grafted into a 5-day lethal rat. When the graft was removed after 10 days' growth, its histological structure was normal. (Azan;  $\times 45$ .)

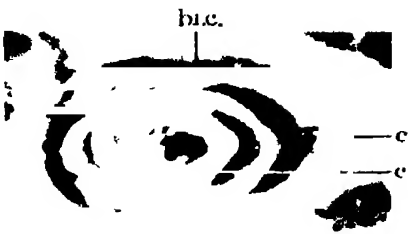
FIG. 12. Transverse section of a (control) costal cartilage from a 3-day lethal rat. (Azan;  $\times 45$ .)

FIG. 13. Transverse section of a costal cartilage removed from the opposite side of the same 3-day lethal rat as that from which the rib shown in fig. 12 was taken. This cartilage was grafted into a normal 14-day rat and fixed after 50 days' growth. The lethal abnormalities have advanced greatly during the period of implantation. Cf. fig. 12 and note the great increase in diameter, the much greater size and density of the pericellular capsules and the very large central area of degeneration. (Azan;  $\times 45$ .)





1



4



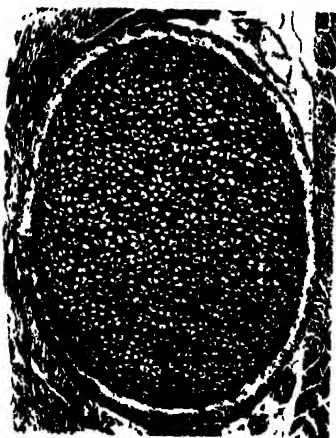
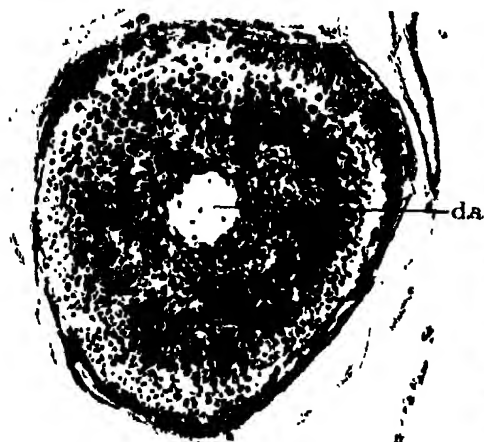
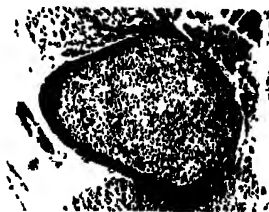
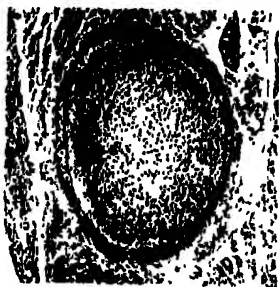
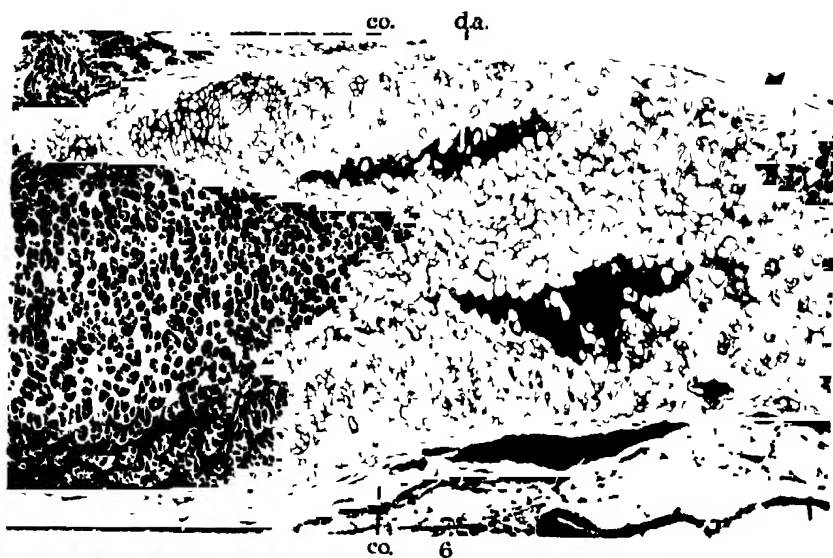
5



2



3





11



12



14



13



15



16



FIG. 14. Transverse section of a costal cartilage removed from the same 3-day lethal rat as that from which the ribs shown in figs. 12 and 13 were taken. The cartilage was grafted into a normal 14-day rat and fixed after 7 days' growth. Note the osteoid appearance of the new tissue formed by the perichondrium. (Azan;  $\times 180$ .)

FIG. 15. Transverse section of a (control) costal cartilage from a 3-day normal rat, litter-mate to the lethal rat from which the ribs shown in figs. 12-14 were taken. (Azan;  $\times 45$ .)

FIG. 16. Transverse section of a costal cartilage removed from the opposite side of the same normal rat as that from which the rib shown in fig. 15 was obtained. The cartilage was grafted into the opposite side of the same 14-day normal rat as the lethal cartilage shown in fig. 13, and was grown for 50 days. The histological structure of the rib is normal, cf. fig. 13. (Azan;  $\times 45$ .)

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## The association of carcinogenicity and growth-inhibitory power in the polycyclic hydrocarbons and other substances

BY A. HADDOW AND A. M. ROBINSON

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Previous papers (Haddow 1935, 1938*a*; Haddow and Robinson 1937; Haddow, Scott and Scott 1937) described the growth-inhibitory action of many carcinogenic hydrocarbons and the absence of this property in numerous non-carcinogenic compounds of somewhat different structure. The possible implications of this association, concerning the mode of action of cancer-producing substances, have already been discussed (Haddow 1938*b*), but the investigation was extended so as to include certain of the benzantracene homologues which have been found inactive in tests for carcinogenic potency. Since such compounds are of course more closely related to the carcinogenic compounds than many of the non-carcinogenic compounds previously studied, such an investigation represents a rather more searching test of the hypothesis that carcinogenicity is dependent on growth-inhibitory power.

The characteristic inhibitory response, and the technique employed to

TABLE I. CARCINOGENIC AND NON-CARCINOGENIC HYDROCARBONS AND RELATED COMPOUNDS

Each symbol in columns 4, 5 and 6 indicates the result of one experiment, based in the case of spontaneous mouse cancer on a single tumour-bearing animal and for the Crocker mouse sarcoma and Walker rat carcinoma on control and treated groups of 10-14 animals each.

— no inhibition;  $\perp$  slight inhibition; + inhibition; \* inhibition with partial regression.

Compound	Carcinogenicity	Dose tested mg.	Inhibitory power against		
			Spontaneous mouse cancer	Crocker mouse sarcoma 180	Walker rat carcinoma 256
1:2-Benzanthracene	+†	10 2 x 30		+	
1'-Methyl-1:2-benzanthracene	Under test	5		—	+
2'-Methyl-1:2-benzanthracene	—	5		—	
3'-Methyl-1:2-benzanthracene	—	5		—	
3-Methyl-1:2-benzanthracene	+	10 2 x 25		+	
3-isoPropyl-1:2-benzanthracene	—	5		—	+
5-Ethyl-1:2-benzanthracene	+	10	+ — — —		
5- <i>n</i> -Propyl-1:2-benzanthracene	+	10		—	
6-Methyl-1:2-benzanthracene	+	10 5	+ $\perp$	+	
6-Phenyl-1:2-benzanthracene	—	2 x 25			+
7-isoPropyl-1:2-benzanthracene	—	10		+ $\perp$	
8-Methyl-1:2-benzanthracene	Under test	10	—	—	
9-Methyl-1:2-benzanthracene	+	10	+ + + $\perp$		
10-Methyl-1:2-benzanthracene	+	10	+ + + + +		
10-Hydroxymethyl-1:2-benzanthracene	+	20	+		
10-isoPropyl-1:2-benzanthracene	—	10	— —		
10-Benzyl-1:2-benzanthracene	—	5	$\perp$ —	—	
2':6-Dimethyl-1:2-benzanthracene	—	5	— — — +		
2':7-Dimethyl-1:2-benzanthracene	—	50		+	—
3':6-Dimethyl-1:2-benzanthracene	—	5		$\perp$	
		10		—	

3':7-Dimethyl-1:2-benzanthracene	5	-			-	
5:6-Dimethyl-1:2-benzanthracene	10	+			+	
	25					
6:7-Dimethyl-1:2-benzanthracene	2 5	+		+		+
	5					
6:7-cycloPenteno-1:2-benzanthracene	10	+		+	+	+
9:10-Dimethyl-1:2-benzanthracene	1	+		* *	+	
	2			+	+	
	2 x 1			+	+	-
	5			+	+	
9:10-Diacetoxymethyl-1:2-benzanthracene	5		Under test	+	+	-
Sodium-1:2-benz-5-anthroate	10		Under test	J	-	
Naphthalene	2 5					
Acenaphthene	10	-				
	8			+		
Pyrene	10			-	-	
	10			-	-	
	12			-	-	
	5					
Triphenylene	2 x 25					
Naphthacene (2,3-benzanthracene)	5	-				
9,10-Dimethyl-anthraccne	10					
1:2:5:6-Dibenzanthracene	5	+	Under test	+	+	+
	10			* *	* *	+
				+	+	+
				+	+	+
				* *	* *	+
	12			+	+	+
				+	+	+
1,2,5:6-Dibenzanthraquinone	10	-				
2'-Methyl-1:2:5,6-dibenzanthracene	10	-				
9-Amino-1:2:5:6-dibenzanthracene	10	+			+	

† 1 epithelioma obtained in 80 mice (Barry *et al.* 1935, p. 323).

TABLE I (continued)

Compound	Carcinogenicity	Dose tested mg.	Inhibitory power against			
			Spontaneous mouse cancer	Crocker mouse sarcoma 180	Walker rat carcinoma 256	
9-Diacetylamino-1:2:5:6-dibenzanthracene	-	10		-		
9:10-Dihydro-1:2:5:6-dibenzanthracene	+	10		+		
9:10-di- <i>n</i> -Butyl-1:2:5:6-dibenzanthracene	+	10		-		
9:10-Dibenzyl-1:2:5:6-dibenzanthracene	+	10		-		
<i>cis</i> -Dimethyl ester of 1:2:5:6-dibenzanthracene-9:10- <i>endo-z</i> $\beta$ -succinic acid	Under test	10	+	-		
1:2:3:4-Dibenzanthracene	-	10	+	-		
		20	-	-		
		10+20	-	-		
1:2:7:8-Dibenzanthracene	+	25				+
1:2-Benzfluorene		10	+	+		
3:4-Benzfluorene	Under test	10	+	-		
		10	-	-		
1:2:5:6-Dibenzfluorene	+	5	-	-		
		10	*	+		+
			+	+		
			+	+		
			+	+		
1:2:7:8-Dibenzfluorene	Under test	25	+	-		+
1:2:5:6-Dibenzacondine	+	10	+	+		
		2	-	-		
		5	*	+		+
			+	+		
			+	+		
			+	+		
		5+2	+	+		
		10	+	+		
		25	+	+		
1:2:5:6-Dibenzphenazine	-	25	-	-		+
		5	-	-		
		6	*	+		
			+	+		
			-	-		
		6+2	+	+		



3:4:5:6-Dibenzcarbazole	+	2 × 0.25 3 × 0.25 10	- + + + + + + + + - - - -	
3:4-Benzpyrene	+			
4'-Hydroxy-3:4-benzpyrene	-	5		+ +
4'-Methoxy-3:4-benzpyrene	-	10		+
3:4:8:9-Dibenzpyrene	+	10		+
3-(N')-4-Pyridinopyrene	Under test	10	+ +	
		5	-	
2':3'-Naphtha-3:4-pyrene	-	10	+ + + -	+
3:4-Benzphenanthrene	+	10		-
2-Methyl-3:4-benzphenanthrene	+	10	+ + + + +	+
			- - - - -	
3:4:5:6-Dibenzphenanthrene	-	10	-	
Methylcholanthrene	+	12	+	
Dehydronorchole	-	10		-
		2 × 25		
Phenanthra-acenaphthene	+	10	-	- -
Acenaphthanthracene	+	10		+
		12	+ + +	
		2 × 12	+	
Anthanthrene	Under test	10		-
Styryl 430	+	5	+ - -	
		2 × 5	+ + +	
			- - - -	
		10	+ + + + +	
		2 × 10	+	
		20	- -	
		3 × 10	+ + +	
		4 × 10	+	

† Pure specimen gave 2 papillomata only.

TABLE II. MISCELLANEOUS SUBSTANCES

Each symbol in columns 3 and 4 indicates the result of one experiment, based in the case of spontaneous mouse cancer on a single tumour-bearing animal and for the Crocker mouse sarcoma on control and treated groups of 10-14 animals each.

- no inhibition;  $\perp$  slight inhibition; + inhibition;  
\* inhibition with partial regression

Compound	Dose tested mg.	Inhibitory power against	
		Spontaneous mouse cancer	Crocker mouse sarcoma 180
Oestrone benzoate	8	$\perp$ - -	
$\beta$ -Indole acetic acid	1.25	-	
	2.5	-	
	5	$\perp$ -	-
	10	- -	
$\beta$ -Indole propionic acid	5		-
Phenylacetic acid	10		-
Sodium fluorene-9-acetate	10	-	
$\alpha$ -Naphthalene acetic acid	5		-
Sodium 1:2-benzanthracene-5-acetate	5	-	
Sodium 6-methyl-1:2-benzanthracene-5-acetate	5	+ + $\perp$ - -	
Benzaldehyde	10†	- -	
Benzidino	2.5	-	-
Aniline nitrate	5		-
1:3:5-Triphenylbenzene	10		- ‡
Aminoazobenzene	2.5	-	
4'-Amino-2:3'-azotoluene (carcinogenic)	5		-
2'-Amino-4,5'-azotoluene	10	- -	
$\alpha$ -Naphthoic acid	10	-	
$\beta$ -Naphthoic acid	5		-
	10	- -	
$\alpha$ -Naphthol	10	-	-
	2 x 10	$\perp$ $\perp$	
1-Amino-2-naphthol-4-sulphonic acid	5		-
Toluene-azo-2-naphthol ( <i>ortho</i> )	5		-
Sodium- $\beta$ -naphthoquinone sulphonate	5		-
$\alpha$ -Naphthylamine	2.5	-	-
$\beta$ -Naphthylamine	3	$\perp$	
	2 x 2.5	-	
	5	+ -	
	10	$\perp$	+

† Emulsion in sesame oil.

‡ Also negative in 25 mg. doses with Walker rat carcinoma 256.

TABLE II (continued)

$\beta$ -Naphthylamine hydrochloride	6 x 0.5	—	
	7 x 0.5	— —	
	2.5	+	
	3	—	
	5	* — —	
	5 + 2.5	—	
$\beta$ -Naphthylamine sulphate	3	⊥ —	
Benzene-azo- $\beta$ -naphthylamino	5	—	—
<i>o</i> -Toluene-azo- $\beta$ -naphthylamino	5		—
Naphthalene-azo-naphthylamino 1:1:4	5		—
	10	—	
Naphthalene-azo-naphthylamino 2:1:2	5		—
	10	—	
$\beta\beta$ -Dinaphthyl	10	— —	

detect it, have been described in detail in the papers mentioned. The present account concerns the results obtained with 96 compounds in 348 experiments using as test object the Walker rat carcinoma 256, the Crocker mouse sarcoma 180, or spontaneous mammary carcinomata in the mouse.

Table I summarizes the results with carcinogenic and non-carcinogenic hydrocarbons and derivatives, and with styryl 430, the carcinogenicity of which was described by Browning, Gulbrandsen and Niven (1936). Table II refers to miscellaneous substances of less direct importance but including several (e.g. oestrone benzoate,  $\beta$ -naphthylamine, *o*-amino-azo-toluene) of cognate interest. In these tables each symbol represents the result of a single experiment. In the case of spontaneous mouse cancer this was assessed from a comparison of the rate of tumour growth, before and after administration of the compound under test, in a single tumour-bearing animal (Haddow 1938*a*). In experiments with the Crocker mouse sarcoma 180 and the Walker rat carcinoma 256 the result was determined by statistical comparison of the mean tumour weight from control and treated groups, each containing 10-14 animals, after an average period of 21 days from tumour implantation and administration of the compound (Haddow and Robinson 1937). With a few exceptions mentioned later in the text, assessment of carcinogenicity in Table I is based on the published results of Cook, Hieger, Kennaway and Mayneord 1932; Cook 1932; Barry, Cook, Haslewood, Hewett, Hieger and Kennaway 1935; and Bachmann, Cook, Dansi, de Worms, Haslewood, Hewett and Robinson 1937.

## DISCUSSION

The sum of results for twenty-eight compounds of proved carcinogenicity (carcinogenic activity + in Table I) and twenty-six non-carcinogenic compounds (carcinogenic activity - in Table I), together with fourteen compounds not given in Table I but tested by Haddow and Robinson (1937),\* is shown in Table III.

TABLE III

	Number of experiments in which result was		
	Inhibition	Weak inhibition	No inhibition
Carcinogenic compounds (34)	148	9	23
Non-carcinogenic compounds (34)	16	7	63

Thus, omitting results classed as weak inhibition, 86.5 % of 171 experiments with carcinogenic substances showed inhibition, while 79.7 % of seventy-nine experiments with non-carcinogenic compounds gave no trace of inhibition: the association is undoubted and extremely significant ( $\chi^2$  circa 100).

It is believed that this finding has immediate importance with reference to the mode of action of carcinogenic substances (see Haddow 1938*b*). Attention must, however, be directed to instances where tumour-producing compounds were not found to possess inhibitory properties (e.g. 5-ethyl-1:2-benzanthracene, 2'-methyl-, 9:10-di-*n*-butyl- and 9:10-dibenzyl-1:2:5:6-dibenzanthracene, phenanthra-acenaphthene). The weakly carcinogenic compound 3:4-benzphenanthrene, which is of special importance on account of its divergence in structure from 1:2-benzanthracene, was found to have no inhibitory action on the growth of the (rocker sarcoma 180, while its actively carcinogenic 2-methyl-derivative proved inhibitory both towards this tumour and spontaneous mammary cancer in the mouse.

On the other hand, a number of apparently non-carcinogenic compounds gave definite indications of inhibitory activity (e.g. 4'-hydroxy- and 4'-methoxy-3:4-benzpyrene, 2', 3'-naphtha-3:4-pyrene). At one stage of the investigation the list of such compounds included 3-methyl- and 7-methyl-

\* 4-Methyl-, 7-methyl- and 5:6-cyclopenteno-1:2-benzanthracene, sodium-1:2:5:6-dibenzanthracene-9:10-*endo- $\alpha\beta$* -succinate, 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene, and 3:4:5:6-dibenzacridine, inhibitory and carcinogenic; anthracene, phenanthrene, 1:2-cyclopenteno-phenanthrene, 1-keto-1:2:3:4-tetrahydrophenanthrene, dodecahydro-1:2-benzanthracene, perylene, 1:9-benzanthrone and diphenylene oxide, non-inhibitory and non-carcinogenic.

1:2-benzanthracene, acenaphthanthracene and 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene, but later tests for carcinogenicity resulted in the production of tumours as follows: acenaphthanthracene: 2 epitheliomata, 1 papilloma; 3-methyl-1:2-benzanthracene: 10 sarcomata, 1 epithelioma; 7-methyl-1:2-benzanthracene: 4 sarcomata (all in mice, personal communication from Professor Kennaway); 7-methyl-1:2-benzanthracene: 1 mouse sarcoma (Shear 1938*a*); 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene: 4 sarcomata in rats (Bachmann and Bradbury 1937). Such evidence obviously affords additional support to the correlation under discussion. With regard to the carcinogenic activity of 3-methyl-1:2-benzanthracene, it is of interest that Shear (1938*b*) obtained sarcomata in mice following the subcutaneous injection of crystals of both 3-hydroxy- and 3-methoxy-1:2-benzanthracene.

It is obvious that other anomalies may conceivably be due (1) to experimental error, (2) to individual variability in the test object, and (3) to the possibility that certain compounds still classed as non-carcinogenic may possess traces of carcinogenic activity. It is also important to recall that there is no absolute standard of carcinogenicity, and that the assessment of this property is derived chiefly from experiments upon the skin and subcutaneous tissues of the mouse.

Special significance is to be attached to the inactivity of those non-carcinogenic monomethyl and dimethyl derivatives of 1:2-benzanthracene containing a methyl group attached to the angular ring. This is in marked contrast with the pronounced inhibitory activity of such (carcinogenic) compounds as the 5:6- and 6:7-dimethyl- and cyclopenteno-benzanthracenes and the 9-, 10- and 9:10-dimethyl-benzanthracenes.

In Table II may be noted the negative results of administration of small doses of *o*-amino-azo-toluene which, although capable of producing hepatoma in rats (Yoshida 1934 *a, b*), has not so far led to the appearance of tumours locally on injection (Nakahara and Fujiwara 1937). Of more positive interest are the irregular results obtained with  $\beta$ -naphthylamine, one of the bases believed to be responsible for the occurrence of papilloma and carcinoma of the bladder in dye-workers (see Hueper and Wolfe 1937; Hueper, Wiley and Wolfe 1938).

In view of the numerous recent reports of the induction of plant tumours by the phytohormones (e.g. Hamner and Kraus 1937; Link, Wilcox and Link 1937; Brown and Gardner 1936, 1937), the finding of Zimmerman and Wilcoxon (1935) that acetic acids derived from the hydrocarbons naphthalene, anthracene and fluorene are capable of causing typical hor-

mone reactions in plants, and the description by Leonian and Lilly (1937) of growth-inhibitory effects due to heteroauxin, several experiments were carried out with indole-acetic acid and related substances. The results obtained were however mostly negative, except in the case of 6-methyl-1:2-benzanthracene-5-acetic acid.

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#### SUMMARY

The results are given for 348 experiments in which 96 compounds were tested for their action on the growth of the Walker rat carcinoma 256, the Crocker mouse sarcoma 180, or spontaneous mammary cancer of the mouse. 86.5% of 171 experiments with 34 carcinogenic substances resulted in growth inhibition, while 79.7% of 79 experiments with 34 non-carcinogenic compounds gave no trace of inhibition. There is therefore a close statistical association of the biological properties of carcinogenicity and growth-inhibitory power.

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## Adrenaline and muscular exercise

BY F. C. COURTICE, *Beit Memorial Research Fellow,*

C. G. DOUGLAS, F.R.S. AND J. G. PRIESTLEY

*From the Physiological Laboratory, Oxford*

*(Received 27 March 1939)*

The mobilization of liver glycogen under the influence of adrenaline has generally been regarded as a factor which may be of importance during muscular exercise, since it may facilitate the transference of glucose to the active muscles. It is, however, by no means clear whether or not this will result in an enhanced use of carbohydrate as a source of energy in the muscles. Dill, Edwards and de Meio (1935) found that a subcutaneous injection of 0.5–1.0 mg. adrenaline during muscular work increases the respiratory quotient and the concentration of glucose and lactic acid in the blood, and concluded that the rise of quotient is mainly due to an increase in the proportion of carbohydrate oxidized. We ourselves (Courtice, Douglas and Priestley 1939), after following in detail the behaviour of the respiratory quotient after the administration of adrenaline and insulin to the resting subject, felt doubtful whether this conclusion was justified, and we have now made a further series of observations on the influence of the subcutaneous injection of adrenaline during muscular exercise.

We have followed the same general procedure as in our previous experiments, using the same methods for the determination of the respiratory exchange, the concentration of glucose and lactic acid in the blood, and the  $\text{CO}_2$  combining power of the blood. The subject was always in the post-absorptive state. After making preliminary determinations of the respiratory exchange and taking blood samples whilst at complete rest reclining in a deck chair, continuous work was done at a steady rate on a Krogh electric brake bicycle ergometer for a period of  $2\frac{1}{2}$ –3 hr., conditions being kept comfortable by the draught from an electric fan. We restricted our observations to either light or moderate work so that there should be no risk of the added complication of the accumulation of a material excess of lactic acid in the blood as a result of the mere muscular activity, as well as to avoid fatigue since neither of the two subjects, Courtice and Douglas, were in athletic training. We felt, too, that any change in the respiratory quotient caused by adrenaline might become relatively insig-

nificant, and therefore more difficult to interpret, if the total respiratory exchange were greatly exaggerated. Light work was equivalent to 210 kg.m./min., involving an oxygen consumption during the first half hour of about 680 c.c./min. in Courtice and 750 c.c. in Douglas, or rather more than thrice the resting metabolism, and moderate work was equivalent to 700 kg.m./min., involving an oxygen consumption during the first half hour of about 1540 c.c./min. in Courtice, or seven times the resting metabolism. Adrenaline chloride (Parke, Davis and Co., 1 : 1000 solution) was injected subcutaneously into the upper arm after the work had been in progress for half an hour.

Figs. 1 and 2 show the results in Courtice and Douglas respectively of the injection of 0.5 and 1.0 mg. adrenaline during muscular work of 210 kg.m./min., a control experiment in which no adrenaline was administered being given in each case. Fig. 3 shows the results on Courtice of the injection of 0.5 and 1.0 mg. adrenaline during muscular work of 700 kg.m./min.

In the control experiments shown in figs. 1 and 2 the blood-sugar concentrations show a slight tendency to fall, particularly in the last hour of the work, but apart from this there is no striking alteration. The lactic acid concentration in the blood remains unchanged at the preliminary resting level, and there is at most but a trivial change in the  $\text{CO}_2$  combining power of the blood. In Courtice there is no significant alteration of the respiratory quotient when the work begins, though the quotient falls very gradually throughout the work period. In Douglas there is an immediate rise of the quotient when the work begins, and this is succeeded by a distinct fall in the first half hour: after this the quotient shows a tendency to fall gradually, though slightly, in the remainder of the work period. With work of 700 kg.m./min. Courtice showed a rise of quotient in control experiments during the earlier stages of the work similar to that shown by Douglas with work of 210 kg.m./min. In one such control experiment the work caused no change in the lactic acid concentration in the blood, but in another there was a slight increase in this concentration during the first half hour similar to that shown in fig. 3 before the injection of adrenaline, and the lactic acid concentration remained at this higher level for the rest of the work period. In fig. 3, therefore, the lactic acid excess due to adrenaline must be regarded as superimposed on the small increase of lactic acid which is liable to be formed in this subject with this degree of work on the ergometer.

In the experiments in which adrenaline was administered the course of events up to the moment of injection (at the end of the first half hour's

work) is the same as in the controls. As a result of the injection there is a rapid increase in the concentration of lactic acid, and a fall in the  $\text{CO}_2$  combining power, of the blood, followed by a steady fall in the concentration of lactic acid and rise in the  $\text{CO}_2$  combining power. In one experiment on Douglas (fig. 2) the lactic acid concentration in the blood is still above

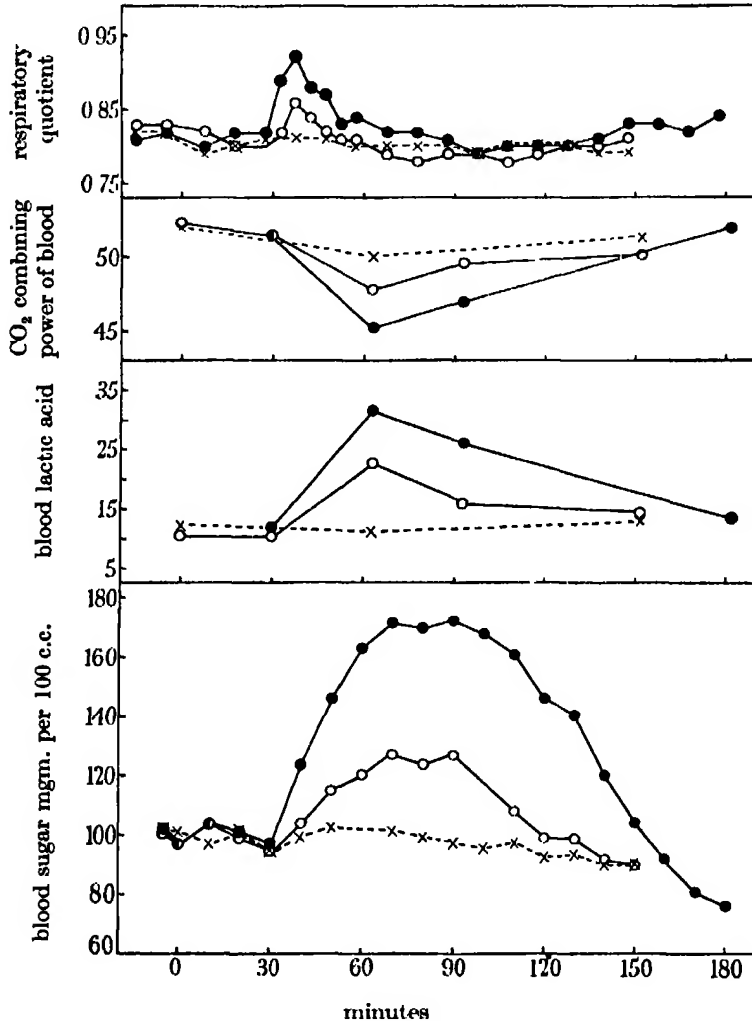


FIG. 1. Subject Courtice. Effect of subcutaneous injection of adrenaline on the blood sugar, blood lactic acid (mg./100 c.c.),  $\text{CO}_2$  combining power of the blood at 40 mm.  $\text{CO}_2$  pressure (in c.c.  $\text{CO}_2$ /100 c.c.), and respiratory quotient. Work of 210 kg.m./min. Work began at 0 min. and adrenaline was injected at 30 min. x Control—no adrenaline. O 0.5 mg. adrenaline. ● 1.0 mg. adrenaline.

its normal value when the experiment stops: in the other experiments both blood lactic acid and  $\text{CO}_2$  combining power had returned to the resting level by the end of the experiment.

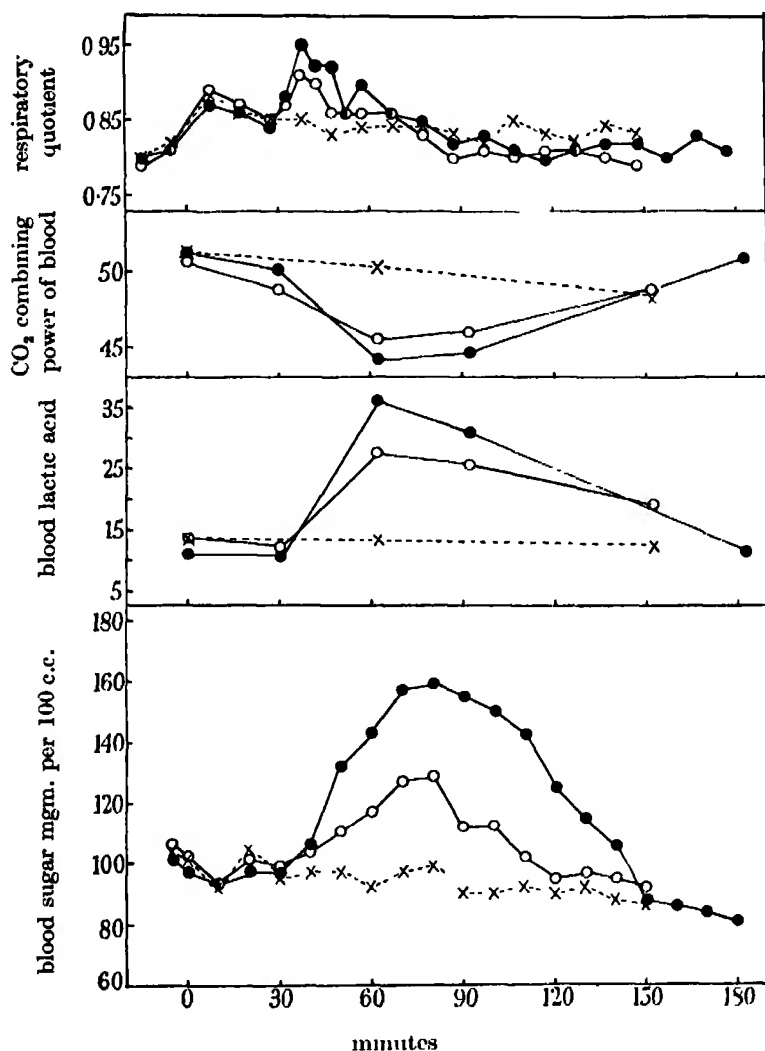


FIG. 2. Subject Douglas. Work of 210 kg.m./min. For description see fig. 1.

x Control—no adrenaline. O 0.5 mg. adrenaline. ● 1.0 mg. adrenaline.

Bang (1936) has shown that in mild or moderate exercise excess lactic acid is actually formed during the first few minutes of muscular work and that this excess subsequently disappears as the work continues, so that a little later on no more lactic acid may be found in the blood than that

which is always present in the subject during rest: moreover, the rate of disappearance of this initial excess of lactic acid is apparently uninfluenced if the subject ceases work and resumes rest. Comparison of our present

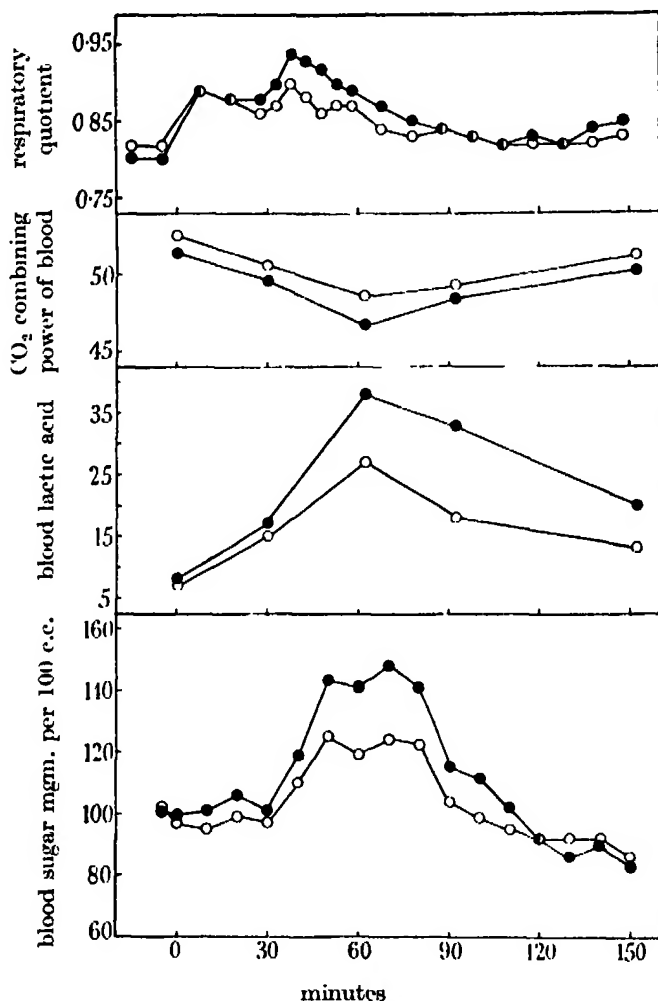


FIG. 3. Subject Courtice. Work of 700 kg.m./min. For description see fig. 1.  
○ 0.5 mg. adrenaline. ● 1.0 mg. adrenaline.

series of experiments with those depicted in figs. 7 and 8 of our previous paper, when the subjects were at complete rest, shows that after the injection of 0.5 mg. adrenaline the concentration of excess lactic acid in the blood mounts to about the same value, and that this excess disappears at about the same rate, in the two cases. The removal of excess lactic acid

formed as a result of adrenaline injection can therefore be as rapid a process during moderate muscular work as during rest. The injection of 1.0 mg. adrenaline causes a more pronounced increase in the lactic acid concentration in the blood, but the excess is subsequently removed during the muscular work at a rate comparable with that found with the smaller dose. The  $\text{CO}_2$  combining power of the blood varies in correspondence with the changes in lactic acid content, falling as the lactic acid concentration rises and recovering its normal level as the excess lactic acid disappears.

The blood-sugar concentration rises rapidly after the injection of adrenaline, attaining its maximum after about 40 min., and, after maintaining a steady level for a short time, subsides to the control level which is regained 2-2½ hr. after the injection. The effect on the blood-sugar concentration is naturally more pronounced when the dose of adrenaline is increased from 0.5 to 1.0 mg. Comparison with the earlier experiments shows that the initial rise of blood sugar is less, and recovery more rapid, during work than during rest, this difference being accentuated the harder the work.

That the behaviour of the lactic acid concentration in the blood after adrenaline injection should be much the same during work as during rest, while the hyperglycaemia is reduced by exercise, is not surprising. The blood flow through the active muscles will be greatly increased and glucose set free in the liver will presumably be the more rapidly removed from the circulating blood by the muscles, particularly if there is an associated increase in the secretion of insulin. On the other hand, the blood flow through the liver need not necessarily show any great alteration from that found during rest, since it will be in part governed by any compensatory constriction of blood vessels in the splanchnic area that may occur. If all, or most, of the lactic acid formed in the muscles is removed by the liver, as Cori maintains, the rate of removal of excess lactic acid from the blood may thus be little affected by exercise.

In our previous experiments the injection of adrenaline into the resting subject was followed by an increase in the oxygen consumption which was evident for 2 hr. or more. During actual muscular work a similar slight increase in oxygen consumption above the figure shown in control experiments was apparent in Courtice, but only in Douglas after a dose of 1.0 mg. adrenaline. The injection of adrenaline during muscular work is followed by a rapid temporary increase in the respiratory quotient. The highest value is reached after about 10 min., and within about half an hour the quotient has regained its normal level. This rise of quotient occurs whilst excess lactic acid is accumulating in the blood and the  $\text{CO}_2$  combining power of the blood is falling. As we had to withdraw on each occasion

10-15 c.c. of blood by puncture of a vein in the forearm to permit of the determination of the lactic acid concentration and the  $\text{CO}_2$  combining power of the blood, we were compelled to limit the number of venepunctures, and for this reason the first withdrawal of blood after the injection of adrenaline was made after the lapse of 30 min. The lactic acid concentration found at this time does not therefore necessarily represent the true maximum: this may well have been reached at an earlier stage. For this reason it is impossible to correlate the rise of respiratory quotient accurately with the observed changes in the concentration of lactic acid in the blood, but it would certainly appear that a large part, and perhaps the whole, of this change of quotient must be consequent on the rapid increase in blood lactic acid and fall in  $\text{CO}_2$  combining power. If we are inclined to attribute this rise of quotient, at least in part, to an increase in the proportion of carbohydrate to fat oxidized, we have to face the difficulty that the quotient is only above its initial level whilst the blood-sugar concentration is rising and has regained the initial level while the blood-sugar concentration is still maximal, and that the behaviour of the quotient in the later stages of the experiments affords no certain indication of enhanced carbohydrate oxidation in spite of the fact that the blood-sugar concentration remains for long above its normal resting level.

If the rise of respiratory quotient represents the expulsion of  $\text{CO}_2$  as excess lactic acid accumulates and reacts with bicarbonate, it will become less obvious the more severe the muscular work owing to the increase in the total  $\text{CO}_2$  output, unless the excess of  $\text{CO}_2$  expelled increases proportionally to the metabolism. Our results show that for a given dose of adrenaline the resultant rise of quotient is always less during work than during rest, and becomes still smaller if the work is increased in severity. If this explanation is correct we should expect to find evidence of a corresponding retention of  $\text{CO}_2$  at a later stage as the excess lactic acid is removed, and the quotient should fall to an abnormally low level and finally regain a normal level when the whole of the excess has disappeared. It is easy to see this in the resting subject as we have shown in our previous paper, but the case is different during muscular work. Take for instance the experiment on Courtice, shown in fig. 1, when 1.0 g. adrenaline was administered. The respiratory quotient just before the adrenaline was injected was 0.82. In the ensuing half hour, during which six consecutive observations of the total respiratory exchange were made, the quotient was above this figure. Assuming that this rise of quotient was determined solely by the expulsion of  $\text{CO}_2$  derived from the neutralization of bicarbonate by the excess lactic acid formed, the total extra  $\text{CO}_2$  due to this, i.e. the quantity in excess



of that required to give a uniform quotient of 0.82, amounts to 1115 c.c. in this half hour. The excess lactic acid which was present in the blood at the end of this time gradually disappears, and 2 hr. later the lactic acid concentration in the blood was at its original resting level. The elimination of the excess was probably complete half an hour earlier since the respiratory quotient had mounted to a level which remained steady during this half hour at a value slightly above the initial figure. It is fair, therefore, to regard the possible period of  $\text{CO}_2$  retention as limited to the preceding 90 min., during which time the average respiratory exchange per minute was 743 c.c. oxygen and 606 c.c.  $\text{CO}_2$ , giving a respiratory quotient of 0.815. If the  $\text{CO}_2$  output were reduced because during this 90 min. a volume of  $\text{CO}_2$  equal to that expelled owing to the original accumulation of lactic acid, viz. 1115 c.c., had to be retained, an average of about 12 c.c./min., the average quotient would only be 0.017 lower than in the absence of such retention. It is evident that for any given quantity of  $\text{CO}_2$  expelled and subsequently retained the influence on the respiratory quotient of a retention which is spread out over so long a period will become the less conspicuous the greater the respiratory exchange, and that a change of quotient that is obvious during rest may become difficult to detect during muscular work.

We have, moreover, found in our experiments that the calculated excess  $\text{CO}_2$  production (i.e. the amount above that required to give the same respiratory quotient as that prevailing before the administration of adrenaline) during the period of high quotient after the injection varies considerably in the different experiments, even when the dose of adrenaline is the same, as is shown in the accompanying table.

	C.c. excess $\text{CO}_2$ expelled during		
	Rest	Work of 210 kg.m./min.	Work of 700 kg.m./min.
Courties			
0.5 mg. adrenaline	510	385	550
	350	—	—
1.0 mg. adrenaline	740	1115	1485
Douglas			
0.5 mg. adrenaline	1180	580	—
	420	—	—
1.0 mg. adrenaline	720	1540	—
	—	1900	—

This is inevitable when so many variable factors are involved—the rate at which adrenaline is absorbed from the site of injection, which must be influenced by the degree of local vasoconstriction, and the speed with

which it is distributed to the muscles and liver, any factors which may tend to inactivate adrenaline, and the time taken for a balance to be struck between the production of lactic acid in the muscles and its removal by the liver. The smaller the initial excess of  $\text{CO}_2$  that is expelled the less easy will it be to identify any change in quotient caused by subsequent retention.

In spite of the difficulties the experiments shown in figs. 1-3 afford several instances in which the disappearance of the initial high quotient is followed by a further downward trend of the quotient with final recovery to a higher level while the lactic acid concentration in the blood is slowly subsiding. This is shown in both experiments on Courtice in fig. 1 with work of 210 kg.m./min., and in one on the same subject in fig. 3 with work of 700 kg.m./min., but not in the other in the same figure when 0.5 mg. adrenaline was given. In one of the experiments on Douglas, shown in fig. 2 (work of 210 kg.m./min.), in which 0.5 mg. adrenaline was administered, the quotient, after an initial rise, falls to a lower level than that which would otherwise be expected during the work and remains at this level until the end of the experiment, but it will be noted that this was the one instance in which lactic acid was still present in excess in the blood when the experiment was stopped 2 hr. after the adrenaline injection. In the other experiment, in fig. 2, the values for the respiratory quotient during the last half hour are rather too irregular to justify any definite statement.

It seems to us, therefore, that the evidence afforded by these experiments justifies the conclusion that the changes of respiratory quotient which result from a single subcutaneous injection of adrenaline during light or moderate muscular exercise are consequent on the formation and subsequent disappearance of excess lactic acid in the blood, and gives no indication of any increase in the proportion of carbohydrate to fat oxidized in spite of the increased mobilization of liver glycogen and long maintained rise of blood-sugar concentration.

#### SUMMARY

1. The effect of a single subcutaneous injection of adrenaline has been studied in two subjects during light and moderate muscular exercise.

2. Hyperglycaemia caused by a given dose of adrenaline is less during exercise than during rest, while the concentration of excess lactic acid in the blood is much the same in the two cases. The excess lactic acid disappears from the blood practically as rapidly during exercise as during

3. The changes of respiratory quotient, when followed in detail, appear to depend on the accumulation and subsequent disappearance of excess lactic acid, and afford no evidence that there is an enhanced oxidation of carbohydrate in spite of the long maintained rise of blood-sugar concentration.

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## Recovery heat in muscle

By A. V. HILL, SEC.R.S.

*From the Department of Physiology, University College, London**(Received 11 May 1939)*

It has been suggested (Sacks 1938, p. 222) "that the post-stimulation heat (in muscle) merely represents the inertia of the tissue in returning to the resting metabolic level after a burst of activity". It is true that the recovery heat *after a single contraction*, as ordinarily observed, would not mean a very large increment in the resting metabolic rate, and that for single contractions it can be accurately measured only by assuming the resting heat rate as a constant base-line. Single contractions, however, have not provided the only, or indeed the strongest, evidence for the existence of the recovery heat; for example:

(i) Hill (1928*a*, p. 188) showed that if oxygen is admitted to a muscle previously stimulated in nitrogen a large and prolonged heat production occurs: this experiment is regularly given as a class demonstration in this laboratory;

(ii) Hill (1928*a*, p. 185) showed that during and after a long regular series of twitches the heat production in oxygen is considerably, not slightly, greater than it is in nitrogen;

(iii) Bugnard (1934) examined the relation between total heat and initial heat during the steady state due to a regular series of shocks in oxygen. The recovery heat, as usual, was about equal to the initial heat. Its accumulated rate, however, was high, not low as after a single contraction. An amount of recovery heat equal to the initial heat occurred every half minute (the interval between shocks) throughout the steady state. The heat rate during the interval between two shocks was about three times that of the resting muscle.

Equally convincing evidence, however, of the reality of the recovery heat is provided by its quantitative consistency under a variety of conditions. It will be shown that the ratio of total to initial heat is the same in the following circumstances (frog's sartorius, 0° C):

(a) for a *single* isometric tetanus of duration from 2 to 24 sec.;

(b) for a series of isometric tetani during a *steady state*;

and is equal to the ratio of (total heat + work) to (initial heat + work):

(c) for a *single* tetanus with maximum work;

(d) for a series of such tetani during a *steady state*.

Moreover, during a steady state of activity the recovery heat rate may be so great that it cannot very reasonably be attributed to an increase in the resting metabolism.

## METHOD

The present experiments have been made at 0° C on the sartorii of active English frogs. There are several advantages in working at 0° C: (i) no ordinary thermostat compares for constancy of temperature with a well-stirred mixture of ice and water: this constancy ensures a constant base-line; after stimulation of the muscle the galvanometer usually returned to its original position within 1 mm., i.e. within  $6 \times 10^{-5}$ ° C; (ii) at 0° C the vapour pressure of water is very small, and errors due to changes of vapour pressure are insignificant; (iii) at 0° C the muscle retains its heat rather better, since loss of heat by evaporation and conduction through the gas is less; (iv) the processes in muscle occur so slowly at 0° C that they can be analysed with greater accuracy; and (v) at 0° C provided that it has been soaked for a long preliminary period in Ringer's solution to avoid "reversible inexcitability", a muscle gives very constant results for many hours, even during "steady" stimulation (at not too high a rate).

The thermopiles used in the present investigation were the rapid ones recently described (Hill 1937, 1938). They are very well insulated, so that no galvanic effects disturb the base-line. They are "protected", so avoiding

errors due to movement of the muscle. Readings in absolute temperature units are obtained directly by calculation, without calibration by heating the muscle. Compared with other thermopiles they lose their heat rather slowly. Their most important advantage, however, is that the heat loss is precisely exponential, so allowing a simple and exact analysis of the readings. With all the older thermopiles the rate of heat loss followed no simple relation and a laborious numerical analysis, based on a "heating control", was necessary (cf. Hartree 1932). With the present thermopiles, once the maximum deflexion is reached, the rate of heat loss at any moment is proportional to the deflexion at that moment, and a single constant provides an exact correction for heat loss.

This property of the thermopiles is tested, and the constant determined for each muscle, as follows. A high-frequency alternating current is led to the stimulating electrodes at the ends of the thermopile and short-circuited by a Morse key. The key is opened for a short time, and the muscle is heated (but not stimulated). The deflexion is observed every 3 sec. on the scale, time signals being supplied by a Jaquet clock and a telephone. The logarithm of the deflexion is plotted against the time, and gives a straight line. The slope of this line supplies the constant required for calculating the heat loss.

Let the falling deflexion be  $e^{-kt}$ , where  $t$  is in minutes. Plot  $\log_{10} e^{-kt}$  against  $t$ , and measure the time  $t_1$  for the logarithm to decrease by unity; then  $e^{-kt_1} = 0.1$  and  $k = 1/0.4343t_1$ . The usual value of  $k$  for a muscle of ordinary size in oxygen at  $0^\circ\text{C}$  on thermopile  $P_6$  is about 2.1. It can be shown that for a production of heat distributed in any manner in time the heat loss up to any moment, to be added to the deflexion at that moment to give the total heat corrected for heat loss, is simply  $k$  times the area of the deflexion-time curve up to that moment. All that is necessary is to plot deflexion (in mm.) against time (in minutes), and to work out the area (in mm.  $\times$  min.). This area multiplied by  $k$  is added to the deflexion and gives the total heat (in mm. deflexion) corrected for heat loss.

It has been assumed that the galvanometer follows the temperature of the muscle exactly. In the present experiments the galvanometer (constructed by Mr A. C. Downing) had a period of 1.28 sec. It follows the temperature of the muscle closely enough, within about 1.2 sec. after the heat is produced.

This property of the thermopile makes it possible to correct simply for two errors in the measurement of the initial heat: (a) heat loss and (b) unequal distribution of heat across the muscle. In most previous work the maximum deflexion of the galvanometer was taken as a measure of the initial heat. The maximum, however, may be reached so late, particularly

if the initial heat production occupies several seconds and with long stimuli, that considerable heat loss may have occurred by then. In the past this error was avoided to some degree by using a slower recording system and comparing the deflexion with that artificially produced by heating the muscle for the same time as the stimulus (cf. Feng 1931). Heat loss was approximately the same in both. With the present method (Hill 1938) of direct calculation of the sensitivity the full effect of heat loss occurs. If, however, the deflexion be observed every 3 sec., during and for (say) 15 sec. after the stimulus, and if the correction for heat loss be applied as described above to the curve so obtained, the initial heat is found as the initial constant level of the corrected curve, from which a later slow rise occurs as recovery sets in. It is simpler, and for short contractions nearly as accurate, to plot the logarithm of the deflexion against the time, and to extrapolate the straight line so obtained backwards to the middle of the stimulus (which is about the "centre of gravity" of the initial heat). This can be done very easily with semi-logarithmic paper. For longer contractions it is more accurate to apply the full correction, and that has been done in the present experiments. In this way a sharp distinction is drawn between initial and recovery heat and, for both, allowance can be made accurately for heat loss. Moreover, any inequalities in the distribution of heat across the muscle, either during contraction or in relaxation (cf. Hill 1938, p. 169), are smoothed out in this way, since (i) extrapolation can be done sufficiently accurately from the later readings made at a time when the heat has been uniformly redistributed, or (ii) the constant level of the corrected curve is reached after such redistribution is complete.

The rate of heat loss of a muscle on a thermopile is determined by three factors:

- (a) conduction of heat along the wires;
- (b) conduction of heat through the surrounding gas;
- (c) evaporation of water from the muscle, due to the fact that so long as it is at a higher temperature its vapour pressure is greater than that of the solution in the chamber.

A thermopile  $P_3$ , with constantan-manganin couples, was tested in air and in hydrogen, at 0° C and at 20° C. The same muscle was used in the four cases. The values of  $k$ , the coefficient of heat loss, were as follows:

$$\text{Air: } 0^\circ \text{ C, } k = 2.31; \quad 20^\circ \text{ C, } k = 2.91.$$

$$\text{H}_2: \quad 0^\circ \text{ C, } k = 4.45; \quad 20^\circ \text{ C, } k = 6.15.$$

This allows us to evaluate the importance of the three factors in heat loss. Suppose that  $k$  is the sum of three  $k$ 's, each due to one of the factors (a), (b), (c) above. Then at 0° C in air

$$k_a + k_b + k_c = 2.31.$$

Now at  $0^{\circ}\text{C}$  in hydrogen  $k_a$  will be unaltered,  $k_b$  will be increased about 7 times, and  $k_c$  will be increased in the ratio of the diffusion constant of water vapour through hydrogen to that through air, viz. 3.5 times. Thus

$$k_a + 7k_b + 3.5k_c = 4.45.$$

Now raising the temperature to  $20^{\circ}\text{C}$  increases the thermal conductivity of the metals by about 5%, and that of air and hydrogen by about the same amount. It increases the vapour pressure 3.8 times and the rate of diffusion of water vapour through the gas 1.17 times; and therefore increases the rate of heat loss by evaporation  $3.8 \times 1.17 = 4.45$  times. Hence

$$1.05k_a + 1.05k_b + 4.45k_c = 2.91$$

and

$$1.05k_a + 7.35k_b + 15.6k_c = 6.15.$$

Solution of these equations gives, for air at  $0^{\circ}\text{C}$ ,

$$k_a = 1.90, \quad k_b = 0.28, \quad k_c = 0.133.$$

Thus at  $0^{\circ}\text{C}$  in air thermal conduction by the wire is 7 times as important as conduction through the gas and 14 times as important as evaporation.

For air at  $20^{\circ}\text{C}$  the values become respectively:

$$1.05k_a = 2.00, \quad 1.05k_b = 0.29, \quad 4.45k_c = 0.59; \quad \text{total } 2.88 \text{ (obs. } 2.91).$$

For air at  $30^{\circ}\text{C}$

$$1.075k_a = 2.04, \quad 1.075k_b = 0.30, \quad 8.68k_c = 1.15; \quad \text{total } 3.49.$$

and for air at  $40^{\circ}\text{C}$

$$1.10k_a = 2.09, \quad 1.10k_b = 0.31, \quad 16.1k_c = 2.14; \quad \text{total } 4.54.$$

At higher temperatures the muscle loses heat considerably more quickly, owing chiefly to evaporation.

The above calculation, based on data taken from Landolt-Bornstein's tables, is only rough. It gives, however, a general understanding of these instruments. Similar reasoning would apply to other gases. For oxygen and nitrogen fortunately the values are nearly identical so that heat loss from a muscle in oxygen is practically the same, except at high temperature, as in nitrogen. In  $\text{CO}_2$  the rate of heat loss would be appreciably less.

For thermopile  $P_4$  (palladium-gold iron couples) which has a much lower electrical resistance and correspondingly a higher thermal conductivity, the same muscle gave a  $k$  of 2.86 in air at  $0^{\circ}\text{C}$ . Presumably  $k_b + k_c = 0.41$  was the same as with thermopile  $P_5$ , so that  $k_a = 2.45$ , compared with  $k_a = 1.90$  for thermopile  $P_5$ .

## EXPERIMENTS

### (1) Recovery after a single contraction

Consider first the single isometric tetanic contraction. A pair of frog's sartorii is mounted on one of the thermopiles referred to above, immersed in oxygenated phosphate-buffered Ringer's solution at pH 7.2 (10 mg. P/100 c.c.) and left for 16 hr. at  $0^{\circ}\text{C}$ . The Ringer's solution is replaced

by oxygen, and the smallest stimulus (condenser discharge) for maximal response determined. This was usually about 6 V,  $0.05 \mu\text{F}$ , and the frequency was about 10 shocks each way per second. If necessary the energy in the stimulus (which appears as part of the initial heat) can be allowed for: it is usually negligible, but if precautions are not taken to use a suitable stimulus it may be important.

It is now necessary to wait for 40 min. to allow recovery to be completed from these preliminary stimuli. The zero is then carefully recorded. The clock is started with its 3 sec. audible time signals. The stimulus is set to begin at one of them. The galvanometer deflexion is then read, at first every 3 sec., later at longer intervals, until at 30–40 min. it has returned to zero. The process can be repeated for the same, or a different, duration of stimulus. Successive stimuli give closely similar readings, and the muscle will give constant results all day.

The curves constructed from the readings are corrected for heat loss by the method described above, to give initial and total heat. The initial heat appears as the early constant level reached by the corrected curve soon after relaxation is complete. The recovery heat is represented by the gradual upward movement of the corrected curve from this initial constant level. The time course of the recovery heat, most rapid at first, decreasing in rate gradually to zero at 30–40 min., will be described elsewhere by D. K. Hill for comparison with his observations of the oxygen consumption of muscle (see D. K. Hill 1939). Here total quantities alone will be referred to (see Table I below). For total heat, only the whole area of the curve need be measured, and no analysis is required.

The initial heat of the isometric contraction contains a certain amount of work which is turned into heat in relaxation—work done in stretching the tendon, chain and lever (see Hill 1938). With the Levin-Wyman ergometer (Levin and Wyman 1927) about 40 % of the initial energy may appear as work, and the experiment described above can be made with a single tetanic contraction in which the muscle shortens at a speed designed to give about maximum efficiency. The recovery heat is then compared with the initial *energy* (see Table I).

### (2) *Recovery during a steady series of isometric contractions*

A muscle is subjected to a regular series of maximal stimuli, e.g. a single shock every  $\frac{1}{2}$  min. (cf. Bugnard 1934), or a 1–6 sec. tetanus every 2–5 min. At the end of about 30 min. a steady condition is reached in which the response becomes uniform, and the amount of recovery in each interval is equal to that required for *one* of the contractions. Extremely constant



results are obtained, if desired for hours, in this way. It is found that the rate of recovery heat production, accumulated from all the preceding contractions of the previous 30–40 min., is nearly constant throughout, being slightly greater at the beginning than at the end of each interval, and having a value about 3–6 times the normal rate of heat production at rest. This high value of the recovery heat rate means that a small alteration in the resting heat rate due to activity, i.e. a temporary change in the base-line, would have very little effect on the calculation. At the end of the period of steady stimulation the deflexion gradually returns to its resting zero, which is either the same or nearly the same as that initially observed. In the latter case an interpolated value is used corresponding to the time when the deflexions were observed during steady activity.

During each interval the deflexion was noted every 3 sec. at first, and then less frequently. Usually observations were made throughout ten successive intervals and the means taken. Actually the successive readings were nearly identical. The initial heat was determined from the beginning of the curve by correcting for heat loss in the manner described above, the assumed base-line for this being the continuation of the very gradually falling curve at the end of the interval: the initial heat is then simply the sudden rise of the corrected curve immediately following stimulation. The total heat was taken as the area of the curve, for a single interval, above the base-line determined as above from the initial and final resting readings. This area (in mm.  $\times$  min.) multiplied by  $k$  is compared directly with the initial heat in mm.

If the stimuli were too frequent or too long no steady state would be possible; recovery could not keep pace with initial breakdown. Successive responses would then become progressively less until either (*a*) the muscle failed, or (*b*) the condition became steady with a diminished initial breakdown. When the response remains constant for 40–50 min., after 30–40 min. of initiation, it can safely be assumed that the condition is steady. During the period of initiation the response slightly diminishes in successive contractions until the steady condition is reached. After stimulation is over the deflexion returns in about an hour to its resting value. The whole process can then be repeated exactly, the muscle remaining in a constant condition all day; or other experiments can be made on it.

### (3) *Recovery during a steady series of contractions with maximal work*

An investigation of the maximal mechanical efficiency of frog's muscle required, for comparison with that of human muscle, that the whole process, not merely the initial process, should be taken into account. The

efficiency of human muscular movement has usually been determined during a state of steady exercise, and it was desirable to measure the efficiency of frog's muscle under similar conditions. It has hitherto been assumed that the ratio (work)/(total initial energy) could simply be divided by 2 to give the total efficiency, since the total energy was probably about twice the initial energy. This assumption, which proves to be correct, had no direct evidence to support it for the case of steady exercise in which external work was being done. To get that evidence, for frog's muscle at  $0^{\circ}\text{C}$ , was the origin of the present experiments.

The procedure was similar to that described in (2) above. A muscle was allowed to contract against either (i) an isotonic lever system, with load adjusted to give about maximal work, or (ii) a Levin-Wyman ergometer with speed adjusted to the same end. In either case the work was collected and not reabsorbed by the muscle in relaxation. The stimuli (about 1 sec. tetanus) were repeated every 4–5 min. until a steady condition was reached. The total heat and the initial heat were measured as in (2) above and to each was added the external work, to give total or initial energy.

## RESULTS

The results are given in Table I. We see that in any one experiment there is no significant difference between the values of  $R$  (the ratio of total to initial energy) obtained by different methods. For a single tetanus the mean value of  $R$  is 2.00; for a steady state with isometric tetani it is 2.03; for a steady state with approximately maximal work it is 2.00. It is clear that the recovery heat is about equal to the initial *energy*, whether in single contractions or during a steady state, whether isometric or with approximately maximal work.

Hartree (1928) also found that the ratio of recovery heat to initial energy is the same in isometric contractions as when work is performed.

The value of  $A$  (the ratio of mean heat rate during steady recovery to resting heat rate) depends on the choice of conditions, particularly on the interval between stimuli. It varied in this series from 3.8 to 7, that is to say, the nearly uniform rate of total heat production (disregarding initial heat) during a steady state of activity was 3.8–7 times that of the resting muscle; the recovery heat rate therefore was 2.8–6 times. The recovery heat accompanies the restoration of the muscle to its initial condition, and—accepting the ratio  $R=2.0$ —half the energy does not appear as heat, but is used in restoring the muscle to its initial state from which it can later

TABLE I

$R$  is the ratio of total energy to initial energy.

$A$  is the ratio of mean heat rate during steady recovery to resting heat rate.

$E$  is the inmechanical efficiency, the ratio of work done to total energy, including recovery.

Isometric contractions unless otherwise noted.

	$E$ %	$R$	$A$
Exp. 1. Single, 2 sec. tetanus	—	1.76	—
Steady, twitches every $\frac{1}{2}$ min.	—	1.73	5
Exp. 2. Single, 2 sec. tetanus	—	1.75	—
Steady, 2 sec. tetani every 2 min.	—	1.89	6.5
Steady, twitches every $\frac{1}{2}$ min.	—	1.74	6
Exp. 3. Steady, 1 sec. tetani every 4 min., isotonic	18.4	1.94	7
Exp. 4. Steady, 1.2 sec. tetani every 5 min., isotonic	17.2	1.96	5
Exp. 5. Single, 3 sec. tetanus	—	1.98	—
Single, 6 sec. tetanus	—	2.04	—
Single, 12 sec. tetanus	—	1.99	—
Single, 18 sec. tetanus	—	1.97	—
Steady, 3 sec. tetani every 5 min.	—	2.03	3.8
Steady, 6 sec. tetani every 5 min.	—	1.91	4.4
Exp. 6. Steady, 1 sec. tetani every 4 min., isotonic	16.4	1.96	5
Exp. 7. Single, 3 sec. tetanus	—	2.04	—
Single, 24 sec. tetanus	—	1.99	—
Steady, 6 sec. tetani every 4 min.	—	1.99	5
Exp. 8. Single, 3 sec. tetanus	—	2.34	—
Single, 3 sec. tetanus, Levin-Wyman ergo-meter	16.5	2.15	—
Steady, 1.2 sec. tetani every 4 min., Levin-Wyman ergometer	17.5	2.13	5.5

give out initial energy again. The rate of oxygen consumption therefore due to recovery must have been 5.6–12 times that of the resting muscle, the rate of total oxygen consumption 6.6–13 times.

For a man with resting metabolism  $\approx 250$  c.c.  $O_2$ /min. these would correspond roughly to 1.6 and 3.2 l. of oxygen per minute, vigorous and severe exercise respectively. The time scale of frog's muscle at  $0^\circ C$  is so altered that what would appear to be very mild activity on our scale of time is in fact, compared with its resting metabolism, quite severe.

It will be asked whether the oxygen supply by diffusion was adequate in these experiments during a steady state. The average rate of oxygen consumption during the "steady" experiments (3) to (8) of Table I has been calculated from the total energy liberated, and compared with the maximum oxygen supply calculated from the measured thickness of the

muscle, Krogh's diffusion constant (assumed to be  $1.15 \times 10^{-5}$  at  $0^\circ \text{C}$ ) and the equations given by Hill (1928*b*). The results are shown in Table II. It is clear that the upper limit of oxygen supply was not nearly reached in any of the experiments.

TABLE II

Exp. No.	3	4	5		6	7	8
Oxygen consumption	2.2	1.6	2.1	1.7	1.4	3.0	1.8
Oxygen supply	4.2	5.4	7.1	7.1	5.4	5.9	3.0

The experiments quoted are those of Table I. The oxygen consumption is calculated from the total energy liberated, assuming 1 cc.  $\text{O}_2 \equiv 5$  cal., and expressed in cubic mm. per gram of muscle, per minute. The maximum oxygen supply by diffusion is calculated (in the same units) from the measured thickness of the muscle and the diffusion constant; with the condition that the whole of the muscle is using oxygen at the same rate and has an adequate supply.

We return finally to the original point. Has the recovery heat a real existence? or should it be regarded simply as an altered resting metabolism? Apart from its very consistent behaviour and its relatively constant ratio to the initial heat determined in several ways, the manner in which it can be accumulated during a steady state of activity provides a convincing answer. To explain that the resting metabolism, owing to the "inertia of the tissue", is increased 7 times during a steady state of activity (and could be increased more, since the limit was not reached), is to use words apart from their ordinary meaning.

#### SUMMARY

1. The recovery heat production of frog's muscle has been reinvestigated at  $0^\circ \text{C}$  under a variety of conditions.

2. The ratio of total energy to initial energy is consistently about 2, (a) in single contractions, followed to complete recovery, (b) in a long series of contractions during a steady state, where the accumulated recovery heat is measured in the interval between two stimuli. It is the same when maximal work is done as when the conditions are isometric.

3. During a steady state the rate of recovery heat production is nearly uniform (decreasing only slightly in the interval between two stimuli) and may be several times as great as the resting metabolic rate. The objection that the post-stimulation heat may be due merely to an increase in the resting metabolism is considered.

4. The allowance for heat loss in such experiments is discussed.

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## The reactions of the urinary bladder of the cat under conditions of constant pressure

BY J. MELLANBY, F.R.S. AND C. L. G. PRATT  
*University Laboratory of Physiology, Oxford*

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Many investigations of the activity of the urinary bladder have been made, especially with reference to the process of micturition. From a survey of the literature it appears that a definite distinction between isometric and isotonic conditions as applied to the neuromuscular apparatus of the bladder has not been rigidly observed. The general method of investigation, where the intact animal has been used, has been to make use of a water manometer permitting considerable fluctuations in intravesical pressure to occur as the volume alters. The present experiments constitute an attempt to isolate the conditions under which changes of volume only may occur, and to study the effects. This paper deals with the activity and response of the urinary bladder of the cat to various procedures when changes of pressure are not permitted to occur.

### 1. METHODS

Cats under chloralose or urethane anaesthesia were used for the majority of experiments. Some experiments were carried out on decerebrate cats, and in a few, ether alone was used.

The abdomen was opened in the middle line, and a ligature placed on the urethra, care being taken not to damage the blood supply of the bladder or the *nervi erigentes*. A glass cannula was then tied into the apex of the bladder, securing as little as possible of the bladder wall in the ligature. The cannula was connected by means of pressure tubing to a glass coil immersed in a water-bath, the object of which was to maintain at body temperature any fluid entering the bladder. The heating coil was connected by pressure tubing via a tap to a cylindrical glass reservoir having a cross-sectional area of 120 sq. cm. A diminution of volume of the bladder of 10 ml. therefore caused an increase of hydrostatic pressure of 0.08 cm. of water. Alterations in the volume of air in the reservoir, produced by fluctuation in the volume of the bladder, were registered by a volume recorder with a frontal writing lever arranged for graphic recording. The system was filled with Ringer's solution.

When inserting the vesical cannula, care was taken to keep the urine in the bladder as far as possible, so that the volume of the bladder after the preparation had been set up was generally only slightly less than the volume observed when the abdomen was opened.

After tying in the cannula, the abdomen was closed in layers.

It was found more convenient to tie the cannula into the apex of the bladder in this way than to insert it via the urethra. Experiments were performed using the latter method, and the results indicated that the response of the bladder was not qualitatively altered by the small amount of operative interference necessitated by the insertion of the cannula through its wall. This opinion is corroborated by the results of Macdonald and M'Crea (1930) who state that it is immaterial whether the cannula in the bladder is inserted via the urethra or through the vesical walls.

## 2. THE EFFECT OF VARYING PRESSURE ON THE ACTIVITY OF THE BLADDER

(a) *Volume.* The bladder tends to adopt a constant volume depending upon the hydrostatic pressure to which it is subjected, and in general the higher the pressure the larger the volume of the bladder. No quantitative relation, however, could be established between the hydrostatic pressure, determined by varying the level of the fluid in the reservoir, and the volume adopted by the bladder. In the experiments described in this paper, the pressure was maintained at some fixed level between 10 and 15 cm. of water, except where indicated. Although the volume taken up by the bladder at any given pressure is unpredictable, its response to changes of

pressure is of a definite and orderly nature. When the initial pressure is not too high, the first response of the bladder to raising the pressure is generally a brief diminution in volume. The contraction is quickly followed by relaxation.

(b) *Rhythm*. If the pressure in the urinary bladder is maintained at a constant level, spontaneous small rhythmic fluctuations in volume appear. These are usually regular in amplitude and frequency. The amplitude depends chiefly upon the volume of the bladder. When the volume is large, fluctuations in volume also tend to be large; when the volume of the bladder is small, the fluctuations tend to be correspondingly small. No constant relation between the amplitude of the rhythm and the volume of the bladder was observed. The amount of fluid entering and leaving the bladder with each rhythmic fluctuation in volume usually lies between 2 and 5 ml. when the pressure is 15 cm.  $H_2O$ . The rhythm disappears when the pressure is reduced below about 3 cm.  $H_2O$  or when the pressure is very high. The upper limit is very variable, some bladders maintaining a rhythm at 150 cm.  $H_2O$ , but the majority become immobile, and maximally dilated, at about 70 cm.  $H_2O$ .

The frequency of the rhythm varies considerably. At 15 cm. water it is generally about 5 per min. In one experiment increasing the pressure by raising the reservoir diminished the rhythm in amplitude from a stroke volume of 5 to 1.2 ml. whilst the frequency progressively increased from 6 to 15 per min. As fig. 1 illustrates, these changes are reversible and indicate that the nature of the rhythm is itself a response to the physical conditions, either of tension or of length, or both, to which the muscle fibres of the urinary bladder are subjected.

These characteristic changes in amplitude and frequency of the spontaneous rhythm do not occur after section of the *nervi erigentes*. It is therefore possible that the responses to variations in the hydrostatic pressure are reflex in origin. It is impossible to say whether the responses to change of pressure are due to alterations in tension, or of length of muscle fibres, since the volume of the bladder cannot be altered without altering the hydrostatic pressure.

### 3. THE INFLUENCE OF ANAESTHETICS

The amplitude and regularity of the rhythm produced under the conditions of these experiments depend upon the anaesthetic agent used and upon the depth of anaesthesia. With urethane, rhythmic movements were observed as soon as recording began. With intravenous chloralose,

however, the spontaneous rhythmic movements of the bladder do not appear during the preliminary deep etherization, and are only evident as the effect of the volatile anaesthetic wears off, and anaesthesia is maintained solely by the basal narcotic. If ether is readministered during chloralose or urethane anaesthesia, after the rhythm has become well established, the

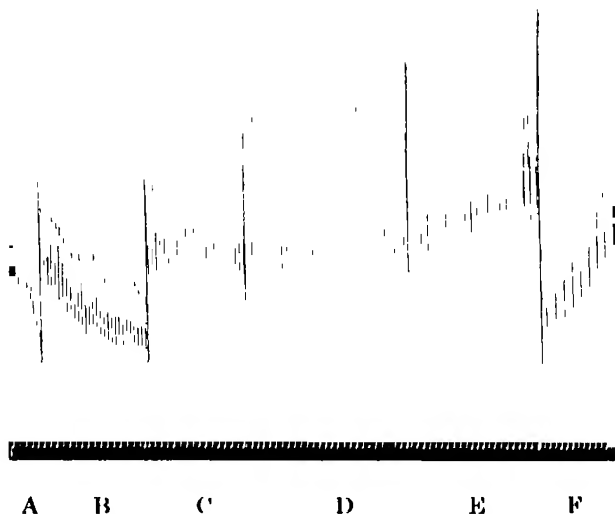


FIG. 1. Diminution of frequency and increase of amplitude produced by lowering pressure. Urethane. A, 43; B, 33; C, 28; D, 23, E, 18; F, 13 cm. H<sub>2</sub>O. Pressure had been raised from 13 to 43 cm. H<sub>2</sub>O in similar increments with the opposite results. Time in 10 sec.

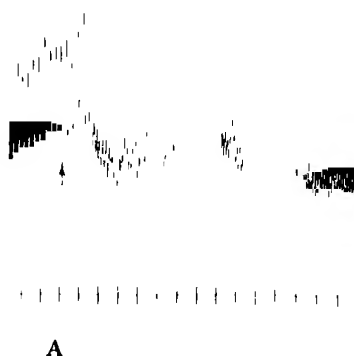


FIG. 2. Isotonic rhythm. Light ether. At A, chloralose 20 mg./kg. intravenously. Time in 30 sec.



rhythm may be again diminished or abolished. At the same time the volume of the bladder considerably increases. When the ether is withdrawn, the activity of the bladder returns to its former level. This may be repeated many times. Under light ether anaesthesia, i.e. when the conjunctival reflex is just present, the bladder may exhibit a well-developed regular rhythm, just as under chloralose anaesthesia. If the administration of ether is maintained at this level and anaesthesia deepened by the intravenous injection of 20 mg./kg. chloralose, the amplitude of the rhythm may be considerably reduced (fig. 2).

When the depth of ether anaesthesia is sufficient to abolish spontaneous rhythm of the bladder, the bladder no longer responds in a characteristic way (see below) to injections of adrenaline or acetylcholine.

The same immobility and lack of sensitivity can be produced by an additional dose of chloralose of 20–40 mg./kg. after the rhythm has already become established under the influence of the usual anaesthetic dose of 75 mg./kg. The ether effect, therefore, cannot be regarded as specific in the chemical sense, but only as an index of the concentration of the toxic agent necessary to produce surgical anaesthesia.

#### 4. THE INFLUENCE OF THE NERVOUS SYSTEM

##### A. *The hypogastric nerve*

(a) *Section.* Section of one hypogastric nerve produces no measurable effects. Section of both, particularly if approximately simultaneously, results in a temporary cessation of rhythmic movements of the bladder. The rhythm recommences in from 2 to 15 min. When it does so it generally exhibits an increase in amplitude and a diminution in frequency. Such a condition is illustrated in fig. 3. This slowing and increased amplitude of the spontaneous rhythm following hypogastric section may be maintained for 5 or 6 hr., but generally it tends to pass off in about 2 hr. The frequency and amplitude then return to their original condition. Section of the hypogastric nerves does not bring about any alteration in the average volume of the bladder, or in its response to changes of pressure imposed upon it by raising or lowering the reservoir. It is therefore evident that if this latter response is reflex in nature, the hypogastric nerves do not constitute part of the reflex arc involved.

(b) *Stimulation.* Stimulation of the hypogastric nerves produces an increase in volume of the bladder, generally with a temporary diminution in amplitude of the rhythm. This inhibition is usually preceded by a brief

contraction, as shown by Elliott (1907). The preliminary contraction can be elicited with a smaller stimulus than can the relaxation.



FIG. 3. Section of hypogastric nerves, while bladder isometric between arrows. Amplitude increased, frequency diminished. Time in 30 sec.

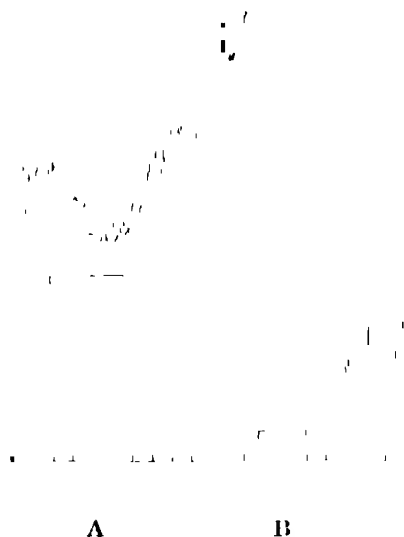


FIG. 4. Effect of hypogastric stimulation at different pressures. A, at 15 cm.  $H_2O$ ; B, at 45 cm.  $H_2O$ . Time in 30 sec.

Using a constant current strength, the relative degree of preliminary contraction and subsequent relaxation depend in part on the pressure maintained in the bladder. When the pressure is low, the preliminary contraction is large, and the subsequent relaxation small (though always much

more prolonged than the contraction). If the pressure is raised and the same stimulus applied, the preliminary contraction may be insignificant as compared with the subsequent relaxation (fig. 4). It was frequently observed that whereas the stimulation of either hypogastric nerve alone produced little or no effect, corresponding stimulation of both together could produce a maximal response. Attempts were made to produce a qualitatively different response by varying the strength and frequency of stimulation, but these were unsuccessful. We were not able, by any form of stimulation of the hypogastric nerves, to produce relaxation followed by contraction, such as follows the administration of certain doses of adrenaline.

### B. *The nervi erigentes*

(a) *Section.* Section of the nervi erigentes always results in considerable dilatation of the bladder (the pressure being fixed) and derangement of the spontaneous rhythm. The rhythm disappears for a time, but shows a tendency to partial recovery in about an hour. Under the acute conditions of these experiments the spontaneous rhythm does not regain complete regularity nor does the bladder completely recover its previous volume (fig. 5). These changes in the posture of the bladder are seen, though in a smaller degree, after section of the nervus erigens on one side only.

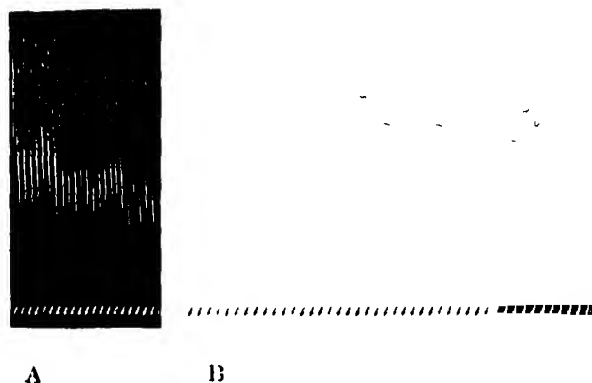


FIG. 5. Partial recovery of rhythm after section of nervi erigentes.  
A, before; B, 2 hr. after. Time in 10 sec.

For a period of several hours after section of the nervi erigentes, the bladder shows greatly increased resistance to the influence of adrenaline and acetylcholine. As already mentioned, the usual changes of amplitude and frequency of the spontaneous rhythm which follow artificially produced changes of hydrostatic pressure cannot be elicited after section of the nervi erigentes.

(b) *Stimulation.* Stimulation of the *nervi erigentes* produces contraction even when the hydrostatic pressure in the bladder is very high. As noted by Langley (1901) and earlier workers, stimulation of the *nervus erigens* on one side produces contraction of that side of the bladder alone. Relaxation of the bladder as a result of stimulation of the *nervi erigentes* was not observed in spite of attempts to produce it by varying the intensity and rate of the stimulation.

## 5. THE ACTION OF ADRENALINE AND ACETYLCHOLINE

(a) *Adrenaline.* The response of the bladder to the intravenous injection of adrenaline depends chiefly upon the dosage employed. Small doses tend to produce contraction only; moderate doses, relaxation followed immediately by contraction; and large doses by relaxation alone. Under surgical anaesthesia with chloralose or urethane or under light ether anaesthesia, the dose of adrenaline required to produce pure relaxation as described by Elliott is very large. Relaxation can nearly always be obtained by intravenous injection of 1 mg. adrenaline. One tenth of this dose generally produces a brief relaxation followed at once by a more marked contraction, both relaxation and contraction temporarily superseding the spontaneous rhythm. Doses of 1/100 mg. usually evoke a well-marked contraction preceded by a small relaxation. In other words it is usually possible to produce the three effects, contraction, relaxation followed by contraction, and relaxation alone, in a single cat, by progressively increasing the dose of adrenaline, although the actual range over which the dose has to be varied differs in individual animals (fig. 6).

Generally speaking, under ether or deep chloralose anaesthesia the relaxation is more easily produced than when the anaesthesia is light, and this is in accord with the results of Macdonald and M'Crea (1930). Moreover the general sensitivity of the bladder to adrenaline also depends on the depth of anaesthesia, so that when the concentration of the anaesthetic agent is high, it may not be possible to demonstrate pure contraction of the bladder with any quantity of adrenaline, the smallest effective dose of adrenaline under these circumstances producing relaxation followed by contraction. A dose of adrenaline small enough to produce contraction alone in the lightly anaesthetized cat may produce no demonstrable effect when the animal is more deeply anaesthetized. The smallest intravenous dose of adrenaline to which the bladder responds, varies therefore with the depth of anaesthesia and the individual cat. The smallest dose observed to produce contraction in this series of experiments was 0.0005 mg.

It might be expected that as a high intravesical pressure favours relaxation on stimulation of the hypogastric nerve, so the inhibitory phase of the action of adrenaline would also be enhanced by increasing the pressure in the bladder. This, however, is not so. The diphasic response of the urinary bladder to a moderate dose of adrenaline is not modified by varying the intravesical pressure over a comparatively wide range.

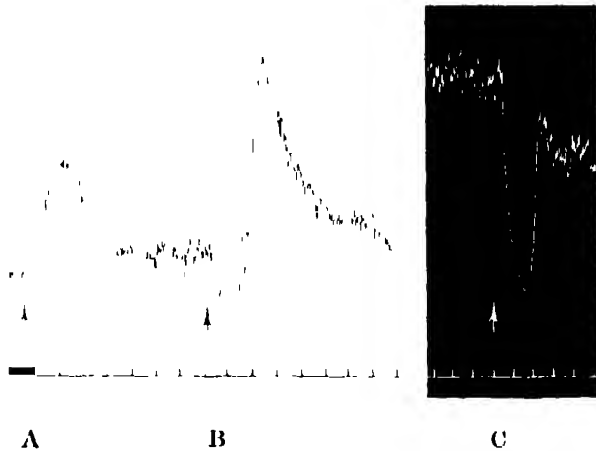


FIG. 8. Effect of varying doses of adrenaline. A, 0.01 mg.; B, 0.1 mg., C, 1 mg. intravenously. Time in 10 sec.

(b) *Acetylcholine*. Acetylcholine produces both contraction and relaxation of the isotonic bladder. The bladder seems to be less sensitive to the action of this drug than it is to the action of adrenaline, and correspondingly greater doses have to be employed in order to obtain a moderate effect. The smallest dose of acetylcholine given intravenously which produced a contraction of the bladder was 0.01 mg. Acetylcholine in doses up to 1 mg. seldom causes a contraction of the bladder of more than 10 ml. in conditions under which 0.01 mg. adrenaline may elicit a contraction equivalent to 30 ml. The effect of acetylcholine may be potentiated, however, by subcutaneous injection of 0.5 mg. of eserine salicylate subcutaneously  $\frac{1}{2}$  hr before the injection of acetylcholine. Under these circumstances the contraction may be as much as 50 ml. in a bladder the total volume of which is only about 75 ml.

The initial contraction produced by acetylcholine differs from the well-known contraction due to electrical stimulation of the *nervi erigentes* in that it may be followed by relaxation. Analysis of the factors influencing this relaxation shows that it is favoured by (i) large doses of acetylcholine and (ii) a high intravesical pressure.

(i) At a given pressure the degree and duration of the secondary relaxation vary, approximately, with the dose of acetylcholine employed (fig. 7).

The initial contraction is never prolonged for more than 30 sec., generally disappearing in about 10 sec., but the subsequent relaxation is generally longer, and several minutes may elapse before the bladder regains its original volume.

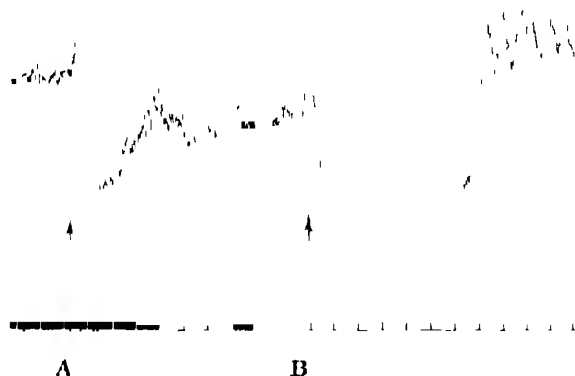


FIG. 7. Relaxation due to the injection of acetylcholine. A, 0.1 mg.; B, 1 mg. at 15 cm.  $H_2O$ . Time in 30 sec.

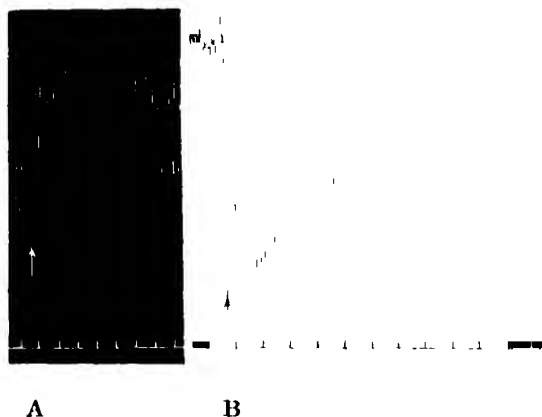


FIG. 8. Influence of hydrostatic pressure on action of 0.1 mg. acetylcholine. A, at 10 cm.  $H_2O$ ; B, at 40 cm.  $H_2O$  (cf. fig. 7). Time in 30 sec.

(ii) The degree of relaxation produced by a given dose of acetylcholine may be modified by varying the hydrostatic pressure. When the pressure is low, relaxation may not be observed, although at a higher pressure relaxation is of considerable degree (fig. 8).

The initial contraction is modified much less than is the relaxation by variations in pressure. It is therefore possible to reproduce the effect of a small dose at a high pressure, i.e. contraction followed by relaxation, by using a large dose at a low hydrostatic pressure.

#### 6. THE BALANCED POSTURAL REACTIONS OF THE BLADDER OF THE CAT

It is evident that the nature of the relaxation produced by acetylcholine differs from the relaxation due to adrenaline, since the former is conditioned by hydrostatic pressure, as stated, whereas the latter is not. Apart from this there is a striking similarity between the effects of the two drugs. Moderate doses of adrenaline and acetylcholine produce relaxation followed by contraction and contraction followed by relaxation respectively. The process of contraction appears to set up the conditions required for relaxation whilst the process of active relaxation leads conversely to a state favourable to contraction. In other words, the reaction to either primary contraction or relaxation produced by the action of these drugs is not "dead-beat" but of a type involving an over-compensation of the original deviation from the resting state.

#### 7. THE ACTION OF ATROPINE AND ERGOTOXINE

Atropine in small doses influences the reactivity of the bladder under constant pressure conditions. Intravenous injection of as little as 0.2 mg. produces an immediate and large dilatation, together with diminution in amplitude of the spontaneous rhythm. The frequency of the rhythm remains unaltered. With doses of 1 mg. or more, the bladder becomes relatively rigid. The amplitude and frequency of the spontaneous rhythm become smaller and smaller and less regular, and neither can be modified by wide variations of hydrostatic pressure. Moreover, altering the pressure from 15 to 65 cm.  $H_2O$  produces no significant increase in volume of the bladder. In such conditions, the bladder no longer responds either to acetylcholine or adrenaline, except to show a temporary cessation in the already small rhythmic movement (fig. 9). The abolition of the sensitivity of the bladder to adrenaline or acetylcholine cannot be looked upon as a specific inhibition of the action of these drugs. Atropine in sufficient doses to produce this state also abolishes the ability of the bladder to contract isometrically.

It is difficult to find a dose of atropine large enough to interfere with the action of acetylcholine, yet not large enough to induce the condition of "fixity" just described.

In a few experiments evidence has been obtained that atropine may abolish not only the contractile response to acetylcholine, but also the contraction due to the action of adrenaline, leaving the relaxation un-

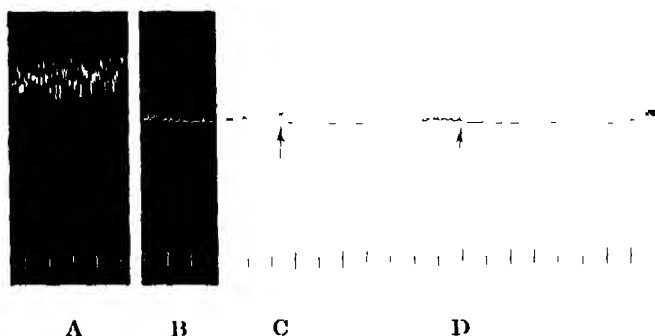


FIG. 9. Immobility after 5 mg. atropine sulphate. A, rhythm before; B, after, atropine; C, 1 mg. acetylcholine; D, 1 mg. adrenaline; smaller doses were equally ineffective. Time in 30 sec.

affected. The possibility that acetylcholine and adrenaline produce contraction of the bladder by similar mechanisms needs more detailed experimental analysis before it can be profitably discussed. Further experiments are being undertaken for this purpose and the results will be made the subject of a further communication.

Ergotoxine in small doses does not interfere with the spontaneous activity of the bladder, but larger doses produce an effect indistinguishable from that of large doses of atropine. Except in doses that produce such fixation of the bladder, ergotoxine does not antagonize the action of adrenaline.

## DISCUSSION

Although the hydrostatic pressure in the bladder has been kept fixed in these experiments, it does not follow that the muscle fibres have been maintained under isotonic conditions. As pointed out by Elliott (1907) the bladder may be regarded as a thin-walled hollow elastic sphere in which the tension of the muscular wall is roughly proportional to the product of volume and hydrostatic pressure, or  $T \propto PV$ . If  $P$  is fixed, then  $T \propto V$ . The existence of regular rhythmic contractions when  $P$  is constant seems to depend upon variations in  $T$  of an oscillatory nature. At the pressure



selected there will be a tendency for the bladder to dilate; as soon as  $V$  commences to increase,  $T$  necessarily becomes greater. The response is a shortening of muscle fibres, reducing  $V$  to its initial value. Repetition of this cycle constitutes the spontaneous rhythm of the bladder at constant pressure.

It is possible to explain the correlation between the amplitude and frequency of the spontaneous rhythm and the hydrostatic pressure in the bladder by assuming that the increment of fibre length (or fibre tension) required to induce the shortening diminishes as the initial tension rises. Increasing the hydrostatic pressure, by increasing  $T$ , then diminishes the extent of the fluctuations of  $V$ . The increased frequency of the rhythm at higher pressure is probably secondary to the diminished amplitude. Since these characteristic adaptations of rhythm and volume to artificially produced changes of pressure do not occur after section of the *nervi erigentes*, they may be reflex in nature. Evans (1936) was able to record, in cats under dial anaesthesia, afferent discharges in the vesical plexus due to distension of the bladder, but apparently did not attempt to correlate these impulses with any spontaneous activity of the bladder.

The rhythm itself is not reflex in origin, for it does not depend on the integrity of the hypogastric nerve or of the *nervi erigentes*. Sherrington (1892) was able to demonstrate an irregular motility of the bladder of the cat after section of all the dorsal and ventral roots, together with section of the cord at the level of the 12th dorsal vertebra. Interruption of the *nervi erigentes* seriously interferes with its regularity and, to some extent, its frequency, but the disturbance is only temporary. The degree and duration of arrhythmia depend largely upon the amount of manipulation involved in cutting the nerves. Any alteration in the spontaneous rhythm is sufficiently accounted for by the general diminution of muscular tone of the bladder following section of the *nervi erigentes*.

Electrical stimulation of either set of nerves to the bladder produces changes of volume during which the rhythmic activity of the bladder is generally superseded by the temporary unidirectional change in muscle tone. In the present experiments relaxation of the bladder as a result of electrical stimulation of the *nervi erigentes* was not seen. Hypogastric stimulation produced both contraction and relaxation. A large number of studies have been made of the innervation of the bladder both from the anatomical and physiological points of view. Gruber (1933), in a comprehensive review of the subject, concludes that the sympathetic and parasympathetic nervous systems each supply both motor and inhibitory impulses to the bladder and that there is no difference between the effects of

the two, except that possibly the parasympathetic nerves carry stronger motor impulses. With that statement the present results agree, but it must be added that the parasympathetic exerts an influence which is more necessary for the maintenance of the spontaneous rhythm than is the influence of the sympathetic nervous system.

Various workers have presented evidence of inhibitory activity in the hypogastric nerve. Langworthy, Reeves, and Tauber (1934), for example, in chronic experiments found that section of the hypogastrics led to a diminished capacity of the bladder, and concluded that the hypogastrics normally diminish parasympathetic activity. Such an effect might account for the striking increase in amplitude of the spontaneous rhythm which often follows section of the hypogastric nerves.

Langley (1901) first noted that adrenaline produces relaxation of the bladder of the cat, and Brodie and Dixon (1904) confirmed this. Elliott (1905) and Dale (1906) stated that adrenaline produces relaxation preceded by contraction. Edmunds and Roth (1920) later came to the same conclusion, and suggested that the earlier workers had failed to observe contraction, because they had employed too great a dose. Edmunds and Roth found that isolated strips from the trigone contract under the influence of very small concentrations of adrenaline, but relax with larger doses. Strips from elsewhere in the bladder were always relaxed. That small doses of adrenaline produce contraction, whilst larger doses cause relaxation of the bladder in an intact animal has been shown by Rose and Deakin (1928), in dogs. None of these investigators, however, examined the bladder under conditions which separated variations in volume from variations in hydrostatic pressure. Under such conditions it is evident that adrenaline has a twofold effect, but it is not yet possible to account for the double action satisfactorily. It is unlikely to be due to opposite types of response from anatomically distinct groups of muscle fibres. The present results indicate that the mechanism which produces contraction under the action of adrenaline is more sensitive to anaesthetic agents than is the mechanism involved in relaxation. In this they are in agreement with those of Macdonald and M'Crea (1930) who found that light ether anaesthesia favoured contraction, whereas deep ether anaesthesia favoured relaxation.

The physiological role of acetylcholine in connexion with the activity of the bladder has been investigated by Henderson and Roepke (1934). Using the perfused bladder of the dog under morphia and choralose they were able to show that acetylcholine appears in the perfusate on stimulation of the nervi erigentes. Perfusion with acetylcholine produces contraction

followed by an increase in postural tonus. The contraction could not be abolished by atropine. However, it is possible to abolish with atropine the action of intravenously injected acetylcholine in the cat.

### CONCLUSIONS

1. The urinary bladder exhibits a regular spontaneous rhythmic variation of volume when the hydrostatic pressure is kept constant.
2. The frequency of the rhythm is increased, and its amplitude diminished, by raising the pressure maintained in the bladder. The effect is reversible.
3. At any given hydrostatic pressure, the bladder assumes a fixed volume. Any attempt to alter its volume also modifies the intravesical pressure.
4. The rhythm may be abolished by deep ether anaesthesia.
5. Contraction and relaxation of the isotonic bladder follow stimulation of the hypogastric nerves. Stimulation of the *nervi erigentes* produces contraction.
6. Section of the hypogastric nerves often increases the amplitude of the rhythm, but never disturbs its regularity.
7. In acute experiments, section of the *nervi erigentes* destroys the regularity of the rhythm.
8. Adrenaline in small doses produces contraction, in moderate doses, relaxation followed by contraction; and in large doses, relaxation alone.
9. The response to adrenaline is not influenced to any extent by the intravesical pressure.
10. Acetylcholine produces contraction or contraction followed by relaxation, the latter especially if the dose be large.
11. The response to acetylcholine is modified by the intravesical pressure. Relaxation is best shown when the pressure is high.
12. Atropine and ergotoxine produce a condition of immobility of the bladder. Experiments depending on the selective blocking of nerve impulses by these drugs are therefore of doubtful value.

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## Morphogenesis and metabolism: studies with the Cartesian diver ultramicromanometer

### I. Anaerobic glycolysis of the regions of the amphibian gastrula

By E. J. BOELL, JOSEPH NEEDHAM AND VERONICA ROGERS

*The Biochemical Laboratory, Cambridge*

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[Plate 6]

#### INTRODUCTION

During the past thirty years our knowledge of the mechanism of morphogenesis has been pushed further forward in the case of the amphibia than in that, perhaps, of any other developmental type. More recently, the facts which have been established by the experimental embryologists have become the subject of biochemical experimentation. The general upshot has been a realization of the extremely close relationships which exist between normal metabolic processes and normal morphogenesis, especially as regards the formation of the primary neural axis in which the action of the evocator in the dorsal lip of the blastopore is involved.

A knowledge of the metabolic processes normally preceding and accompanying the liberation of the primary evocator in the dorsal lip of the blastopore will evidently give us a clearer picture of the exact nature of

the connexion between metabolism and morphogenesis in the gastrula. A morphogenetic hormone, the effect of which must surely be the ordering of protein molecules in cell and tissue architecture, does not arise from nowhere; it has a metabolic origin and metabolic relationships.

Initial steps were made by histochemical methods. The earliest investigation was that of Voss (1931), who found that while the ectoderm of the gastrula gave a strong "plasmal" reaction, there was none to be seen in the roof of the archenteron immediately after invagination. As the substance, plasmalogen, responsible for this reaction, now turns out to be, according to Feulgen, Bersin and Behrens (1938) and Bersin, Willfang and Nafziger (1938), a special type of phosphatide, in which aldehydes of higher fatty acids are combined with glycerol, phosphoric acid and aminoethyl alcohol, it may be that the dorsal lip of the blastopore is the site of considerable changes in lipin metabolism. This, nevertheless, remains an obscure subject.

A much clearer line of work was begun when Woerdemann (1933) demonstrated a similar disappearance of glycogen from the dorsal lip during invagination, and this was put beyond the reach of criticism when Heatley (1935) and Heatley and Lindahl (1937) confirmed it using a reliable microtitration method, based on the techniques of the Carlsberg school. Woerdemann spoke of a "Glykogen-grenze" in the dorsal lip, so sharp was the disappearance of glycogen from the cells as they invaginated. There has been for a long time an impression among the workers on these subjects that carbohydrate metabolism is in some way connected with organizer action. Fischer and Wehmeier (1933) at first claimed that glycogen was the natural evocator, but it was later shown that the activity of glycogen preparations was due to ether-soluble material adsorbed on them, or in loose combination with them (Waddington, Needham, Nowinski and Lemberg 1935), and the claim was later withdrawn (Spemann 1938). The hypothesis that the inactive complex includes glycogen as well as evocator and protein has been put forward (Needham 1936).

In his recent Silliman Lectures, Spemann (1938, p. 244) seems to maintain that the liberation of the evocator in the ventral ectoderm by treatment with heat cannot be the same process as the liberation of the evocator in the dorsal lip of the blastopore by normal metabolism. For this distinction, however, there are no convincing grounds. The fact that a loose complex may be broken down in many different ways is no argument in favour of the existence of more than one complex of uniform composition.

For a long time it has been evident that side by side with direct estimation of substances in presumptive neural plate, archenteron roof, and

ventral ectoderm, we needed a survey of the characteristics of these tissues along the lines of the Warburg technique, measuring the respiration, fermentation, and respiratory quotient. But the technical difficulties in measuring accurately the gas exchange of a piece of tissue weighing, perhaps, only 50  $\mu$ g. dry weight, were very considerable, and in the earlier trials of experiments can hardly be said to have been overcome.

The first investigation was that of Brachet (1934). He compared the respiration of *Rana temporaria* gastrulae in which the dorsal lip had been destroyed by cautery, with others in which a part of the ventral ectoderm had been similarly destroyed, using Fenn microrespirometers and a small type of Warburg manometer. It resulted that the loss of the dorsal lip region entailed a considerably greater diminution of oxygen consumption than the loss of ventral ectoderm. Comparisons of the carbon dioxide elimination by the Saunders pH indicator method on isolated pieces of dorsal lip and ventral ectoderm gave similar results (dorsal lip : ventral ectoderm :: 1.89 : 1.00). The experiments could, however, only be regarded as qualitative, since no measurement of the size of the pieces was made. In a second series of experiments, Brachet (1936) modified the Saunders method to include a Linderström-Lang microtitration, and measured the amount of the isolated pieces of *Discoglossus pinctus* gastrulae by a usual micro-Kjeldahl method giving the total nitrogen. Again the carbon dioxide elimination was 84 % higher in the case of the dorsal lip than in that of the ventral ectoderm. He also made estimations of respiratory quotient by the Meyerhof-Schmitt method, obtaining 1.1 for the dorsal lip and 0.77 for the ventral ectoderm. At the same time, Waddington, Needham and Brachet (1936) measured the oxygen uptake of *Triton alpestris* isolates from dorsal lip and ventral ectoderm in a modified Gerard-Hartline microrespirometer, the dry weight of the pieces being obtained on a microbalance. In this case the oxygen consumption for unit weight proved to be of exactly the same order in the two cases. Finally, Brachet and Shapiro (1937) devised an ingenious apparatus in which the intact gastrula was made the centre of two Gerard-Hartline capillaries, so that the respiratory rate of one hemisphere could be compared directly with that of the other. Using embryos of *Rana sylvatica* a difference of 47 % was found in favour of the dorsal lip hemisphere.\*

\* Since this paper was written, Fischer and Hartwig (1939) have published their results on the oxygen consumption of isolated dorsal lip and ventral ectoderm pieces. They obtained an average excess of 20 % in favour of the dorsal lip, but as their experiments were performed on as many as sixty isolates in relatively large Warburg manometric cups, and lasted as long as 24 hr., it is doubtful whether much significance can be attached to their results.

We shall critically evaluate these results in a later paper in this series, dealing with respiration; here they are only mentioned to outline the position at the time when the present scheme of work was undertaken. We were not satisfied that the micro-manometric methods previously available were really delicate enough for the problem, and we therefore welcomed the proposal of the Cartesian diver method with particular interest. We determined to apply it first to the measurement of anaerobic glycolysis.

### METHOD

The use of the Cartesian diver\* as an ultramicromanometer was first suggested by Linderstrøm-Lang (1937), and further details of the method as used by the Carlsberg school were given in a later communication (Linderstrøm-Lang and Glick 1938). The Danish workers, however, did not elaborate the method for use with minute pieces of tissue.

If a bubble of air or gas is enclosed in a small glass vessel so that it floats within a larger vessel, the buoyancy of the diver will vary according to the pressure imposed on the whole system, and it will sink or rise as this pressure rises or falls. If the pressure is approximately constant, the diver will hover in unstable equilibrium. Conversely, if the gas phase in such a diver is increased or diminished in amount by the process of some chemical reaction inside it, the pressure required to maintain it at a given level will correspondingly rise or fall. In this way the diver is equivalent to a constant-volume manometer if from time to time it is brought to a zero line and the pressure required to do so noted.

The principle of the method is shown in fig. 1. B shows a diver of the type used in the present paper. The tissue and the physiological medium occupy the bottom of the diver bulb, beneath which there is a glass tail to maintain the diver in a vertical position. The amount of glass in the tail is adjusted so that the equilibrium pressure of the diver is fairly near that of the atmosphere. The neck is partly occupied by a drop of oil, so placed that there is a small bubble of air between the oil and the flotation medium. The flotation medium, a strong salt solution giving minimum gas solubility without excessive viscosity, is seen within the diver vessel in diagram A,

\* It appears that the Cartesian diver has nothing to do with Descartes. It was first described by Raffaello Magiotti, a pupil of Galileo, in his *Resistenza certissima dell'Acqua alla compressione dichiarata con vari scherzi in occasione d'altri problemi curiosi* (Moneta, Rome, 1648). Apparently Magiotti published nothing else but this very small book on the incompressibility of water, which he dedicated to Lorenzo dei Medici. The book contains a diagram of the diver, which was no doubt later called "Cartesian" as a synonym for anything "scientific" or mechanical, and remained without application, a philosophical toy, until the present time.

with the diver floating in it. The diver vessel is submerged in an accurately regulated thermostatic water-bath, and connected to a water manometer, the pressure in which can be adjusted (as in a Warburg manometer) by a syringe mechanism. It is convenient to have fine and coarse adjustments.

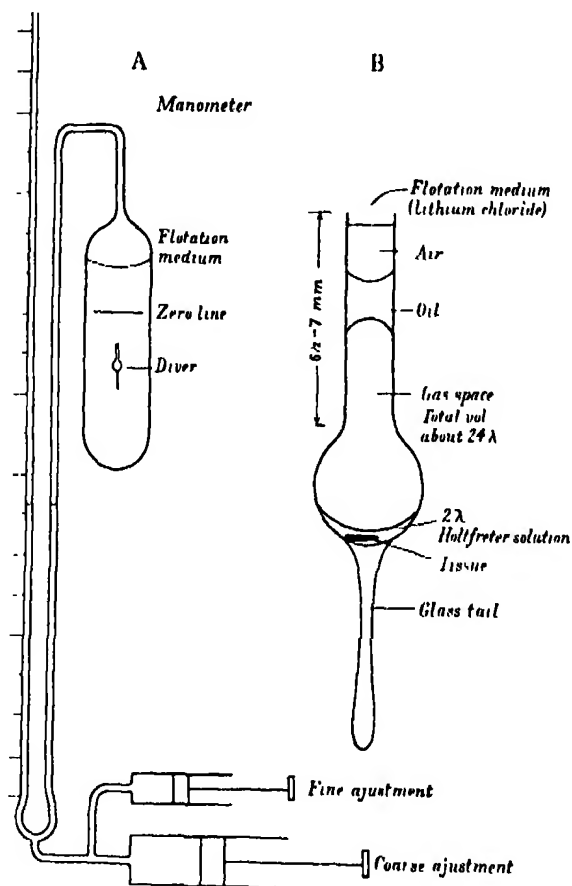


FIG. 1

Readings are accurate to 0.1 or 0.2 cm., and it is therefore possible to measure changes of the order of a millionth of a cubic centimetre of gas. To take a reading, it is only necessary to bring the diver to a temporary standstill at the zero line, and to note the pressure. As will be seen, the Cartesian diver micromanometer has proved of the utmost value for the range of gas exchanges which it was desired to study.



## TECHNIQUE

(1) *Method of making divers*

Divers are made from the uniform bore Pyrex capillary glass tubing supplied under the name "Capillator tubing" by British Drug Houses. The glass is worked over microflames, made from old hypodermic syringe needles cut off straight, against a black background. One end of a convenient length of tubing is first collapsed for a distance of from 6 to 9 mm. This makes the tail. The other end of the tube is then sealed. If now the point of junction between the tube and the solid tail is cautiously heated with a rotatory motion over the microflame, a bulb will automatically blow itself owing to the expansion of air in the tube. The glass neck is then cut off at any desired length. Divers with volumes ranging from below  $10\lambda$  to as much as nearly  $40\lambda$  can be easily prepared in this way.\* As the glass is Pyrex they are remarkably resistant to injury, can even be dropped from some height without harm, and last for use, being cleaned and cleaned again, for many weeks.

(2) *Dimensions of divers*

The divers used in the present investigation, as shown in fig. 1 B, had a total volume of from 20 to  $25\lambda$ , of which  $2\lambda$  were occupied by the physiological medium and the tissue. A length of 1 mm. in the neck is equivalent to a volume of  $1.23\lambda$ , and the oil used was usually 2 mm. in length. The neck length was from 6.5 to 7 mm.

(3) *Method of testing diver buoyancy*

Concentrations of the flotation medium varying by 2% of salt are prepared. A diver is set up, with solution, tissue, and oil, exactly as if for an experiment, and adjusted by removing glass from the end of the tail or by adding glass to it, until it just balances in the normal flotation medium, at atmospheric pressure. It is then emptied and cleaned (see below). By testing it in each tube it is then possible to find a pair of tubes in one of which it just rises (at atmospheric pressure) and in the other of which it just sinks. All subsequent divers required for the experimental series are then tested directly on these two tubes, 5 sec. being allowed for a rise or fall if the column of flotation medium is about 15 cm. long. In this way divers can be so adjusted that not more than 20 cm. pressure of water is required to lift them from the bottom of the diver vessel.

\*  $1\lambda = 1$  cu.mm.

(4) *Flotation medium*

The flotation medium originally employed by Linderstrøm-Lang and Click (1938) was saturated ammonium sulphate. For our purposes, which included the determination of the amount of the tissue present by an ultramicro-Kjeldahl method, and also the determination of the amount of ammonia formed by the tissue during the experimental period, such a salt solution was absolutely inadmissible. We chose as a substitute lithium chloride at the same density as saturated ammonium sulphate. As is shown in Table I, there is not much difference in  $\text{CO}_2$  solubility between the two salt solutions at low concentrations, but on approaching saturation, lithium chloride is a good deal more efficient than ammonium sulphate as a solution in which carbon dioxide is insoluble.

By means of timing the descent of small metal balls through a long column of the two salt solutions at the same density, we satisfied ourselves that the viscosity of the lithium chloride was of approximately the same order as that of the ammonium sulphate.

TABLE I

Salt		Concentration by vol. g./l.	Density	$\alpha_{\text{CO}_2}$
Ammonium sulphate		144	1.0805	0.575
	Sat.	438	1.245	0.235
Lithium chloride		125	—	0.60
		476	1.245	0.127
	Sat.	581	1.302	0.13 approx.

The above data, where not taken from Seidell's Tables, were obtained by direct estimation of carbon dioxide solubility by Dr G. S. Adair.

(5) *Method of calibration*

At the time when the present work was begun, no fuller description of the method existed than the note of Linderstrøm-Lang (1937) in *Nature*. We therefore worked out the calibration independently.

We adopted the principle of comparing the behaviour of divers with that of standard Warburg manometers, using fairly simple reactions of known properties. Thus we studied the autoxidation of cystein, with or without the catalysis of iron (Harrison 1924), for oxygen consumption; and the decomposition of oxaloacetic and acetoacetic acids, with or without the catalysis of aniline (Ostern 1933), for carbon dioxide production. We also studied the anaerobic fermentation of yeast.

Our first observations showed that with quantities as low as a few  $\mu\text{g.}$  of cystein, the autoxidation gave very good linear oxygen uptakes. Fig. 2 shows the autoxidation of 6.3  $\mu\text{g.}$  of cystein in a diver. The end-value was also examined, as shown in fig. 3. 1.0  $\lambda$  containing 3.15  $\mu\text{g.}$  of cystein was

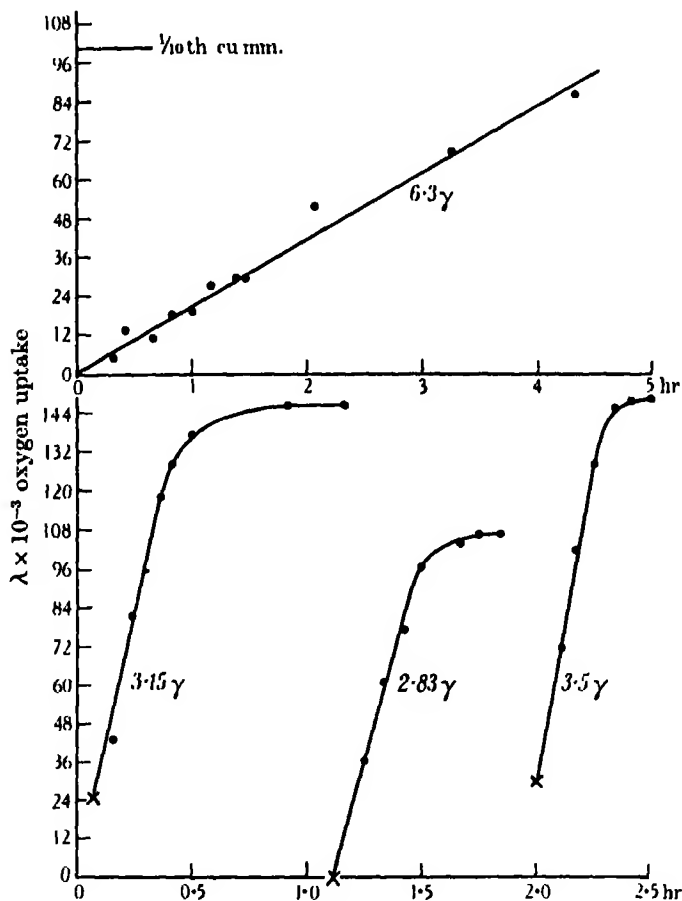


FIG. 2

measured into the diver, and immediately afterwards 0.8  $\lambda$  of an  $\text{FeCl}_3$  solution was also added in the same way from a microburette. This amount contained  $528 \times 10^{-8}$  mg. Fe. The points on the graph represent the readings, and the crosses the moment of mixing, to which the lines are extrapolated back. It will be seen that complete autoxidation was reached in half an hour.

Similar linear graphs were always obtained with yeast suspensions, 1–2  $\mu\text{g.}$  (dry wt.) being used in the divers.

The earlier quantitative comparisons between the values given in the divers and those given in the Warburg manometers were rather rough, for at that time no thermobarometer divers were run, as is necessary for exact work. In the case of cystein oxidation rate, however, the average diver results were 106 % of the Warburg results; in the case of cystein oxidation end-value, they were 99 %, and in the case of yeast fermentation 102 %.

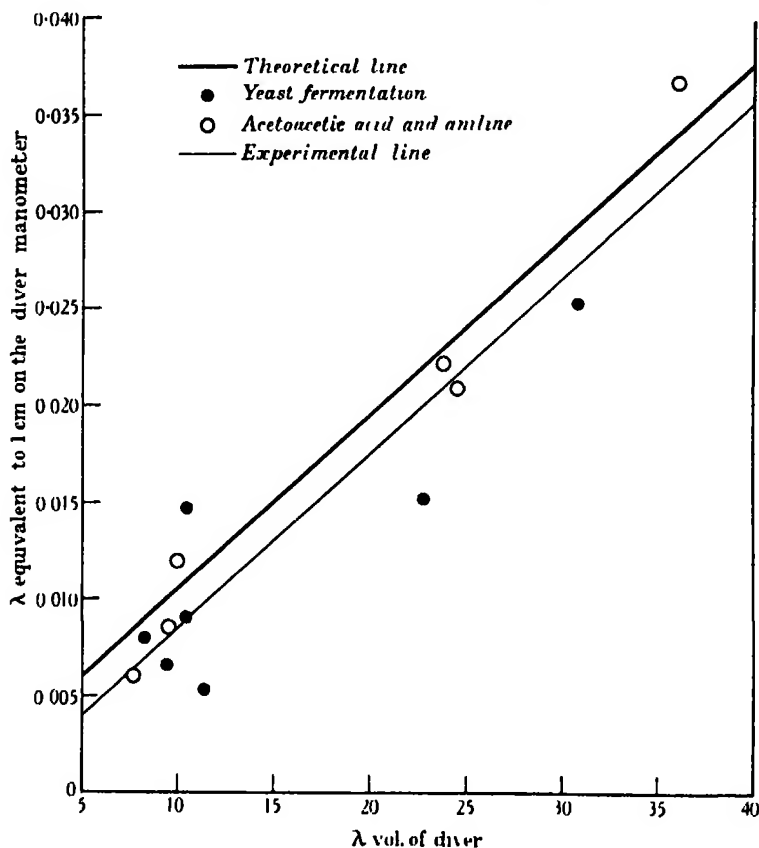


FIG. 3

Since the diver is constantly brought back to its original position at the zero line, it constitutes a constant volume manometer. We therefore considered that a Warburg equation ought to hold good, and we accordingly calculated a theoretical graph relating the diver volume to  $k$  ( $\lambda$  gas change equivalent to an excursion of 1 cm. on the scale of the water manometer). Testing then under well-controlled conditions of thermostatic equilibrium and using thermobarometer divers, we compared the performance of

divers of different volumes with standard Warburg manometers as before. Thus to take a concrete instance:

0.5 c.c. of an acetoacetic acid solution (i.e. 12.45 mg.) was mixed with 0.5 c.c. acetate buffer at pH 5.0, and with 0.2 c.c. dilute aniline emulsion, and made up to 5 c.c. 3 c.c. were measured into a Warburg manometer, where at 17°C in 15 min., 317 cu.mm. carbon dioxide were evolved. 0.001 c.c. (1  $\lambda$ ) was measured into a diver of 9.6  $\lambda$  volume. In 15 min. an excursion of 12.8 cm. on the diver manometer was observed. As 1  $\lambda$  of the solution should give off 0.105  $\lambda$  carbon dioxide, 1 cm. on the diver manometer scale was equivalent to 0.0082  $\lambda$ . From the theoretical equation, a diver of this volume should give a gas change of just under 0.01  $\lambda$  per cm. of the diver manometer.

The results of such experiments are shown in fig. 3 for yeast fermentation and carbon dioxide production from acetoacetic acid catalysed by aniline.

The Warburg equation from which the theoretical line in fig. 3 is drawn, however, is modified to allow for the special conditions in the divers. We take  $V$  to be the space right up to the level of the lithium chloride flotation medium, since the solubility of carbon dioxide in the oil used to seal the neck cannot be neglected. It may be stated at this point that we use as oil a mixture of 50 % acid-free kerosene and 50 % medicinal paraffin oil. From the figures of Kubie (1927) and our own measurements it is seen that carbon dioxide is hardly less soluble in paraffin oil than in water.

TABLE II

	Paraffin oil (24") $\alpha$	Water (20") $\alpha$	Lithium chloride .1 1.245 (19") $\alpha$
Carbon dioxide	0.841	0.878	0.127
Oxygen	0.134	0.031	—
Nitrogen	0.071	0.015	—

It is therefore rather on the relatively slow speed of diffusion of gases through the oil, than on their insolubility in it, that the efficiency of an oil seal must depend.

The diver equation is therefore

$$K_{\text{CO}_2} = - \frac{V_{\text{G}} \times \frac{T}{t} + V_{\text{F}} \times \alpha_{\text{water}}^{\text{CO}_2} + V_{\text{oil}} \times \alpha_{\text{oil}}^{\text{CO}_2}}{P_0 (\text{water})},$$

where  $K$  is the constant of the manometer,  $V_{\text{G}}$  the volume of the gas-space,  $V_{\text{F}}$  the volume of the tissue-medium and tissue,  $V_{\text{oil}}$  the volume of the oil,  $T$  the freezing-point of water (abs.),  $t$  the bath temperature (abs.), and  $P_0$  the normal pressure in mm. of the manometric fluid (in this case

water). From reference to fig. 3 it will be seen that 1 cm. on the diver manometer scale corresponds to a gas change of  $0.006\lambda$  ( $6\lambda \times 10^{-3}$ ) at a diver volume of  $5\lambda$ , and to a gas change of  $0.033\lambda$  ( $33\lambda \times 10^{-3}$ ) at a diver volume of  $35\lambda$ .

The manometer excursion,  $h$ , multiplied by  $k_{\text{CO}_2}$ , gives, of course,  $x_{\text{CO}_2}$ , the actual gas output.

It will be seen from fig. 3 that there is considerable agreement between the theoretical line and the experimentally established points, which, however, are rather better represented by a line parallel with it and a little lower.

A further correction is necessitated by the fact that only the open limb of the manometer is read. The constant obtained by the above formula has therefore to be multiplied by a factor expressing the relation between the levels of the water in the two limbs, and so dependent on the total volume of air intervening between the surface of the flotation medium and the level of the water in the closed limb of the manometer. It was experimentally found that in the plan of the apparatus finally adopted, a rise of 10 cm. in the open limb of the manometer always implied a rise of 6.61 cm. in the closed limb. The equation as finally used, therefore, was as follows:

$$K_{\text{CO}_2} = \frac{(V - 4) \times \frac{273}{293} + 4 \times 0.86}{10350} \times 0.661,$$

since  $V_F$  and  $V_{\text{oll}}$  were  $2.0\lambda$  each, the bath temperature was  $20^\circ \text{C}$ , and the average of the two  $\alpha_{\text{CO}_2}$  factors (see Table II) is 0.86.

From the above it is clear that a modified form of the Warburg equation can be used to calibrate the divers.

The main difference between our procedure and that of Linderström-Lang and Glick (1938) is that we measure the volume of the divers directly by filling them with water from a carefully calibrated Rehberg-Heatley microburette (see later) of  $50\lambda$  capacity, while the Carlsberg workers calculate the volume from an expression involving terms for the weight of the empty diver, the volumes of oil and reaction mixture, the total gas space, and the specific gravities of flotation medium, reaction mixture, glass, and oil. Linderström-Lang and Glick neglect the solubility of gases in the oil seal, but insert a term for the gas bubble between the oil and the flotation medium, although they state that it only affects the result by 1 or 2% and so may be disregarded.

We were able to confirm the results of the Carlsberg workers with air-filled divers. In the absence of serious barometric changes, these divers

will readily remain constant in buoyancy over a period of many hours. If no oil seal is present there is a constant leakage of air into the flotation medium. The same applies to divers filled with nitrogen/ $\text{CO}_2$  mixture (95 %  $\text{N}_2$ , 5 %  $\text{CO}_2$ ), though in this case a slight fall was always observable. This does not agree with fig. 3 of Linderström-Lang and Glick's paper. As will be seen later, we were able to abolish completely this tendency to fall by previously saturating the lithium chloride flotation medium with the gas mixture. Nor did our experiences with pure  $\text{CO}_2$  in the divers quite agree with theirs. Thus, the loss from our divers, if filled with pure  $\text{CO}_2$  without oil seal, was very much greater than that reported by them. It was also correspondingly greater if the oil seal was present. It is possible, though not perhaps very likely, that this difference is due to a slightly larger diameter of neck in our divers. The employment of divers as thermobarometers is essential especially if, as was often the case in the present work, the total excursion of the manometer during the experiment is under 10 cm.; during the experimental period the thermobarometer divers may vary by as much as 2 cm. in 2 hr.

We were still not satisfied, however, that the loss of carbon dioxide through the oil seal could be neglected in glycolysis experiments, and we accordingly made a number of special investigations to check this factor. The paper of Kubie (1927), already referred to, shows that the greatest caution should be exercised in the matter of oil seals. Moreover, Lallemand (1932) made determinations of oxygen by the Winkler method in evacuated water standing in air under a layer of paraffin oil 10–40 mm. thick. In the absence of oil, the normal state of saturation with oxygen is nearly reached after 25 hr., and the rate is very much faster in the initial stages, so that the half-way point is reached in under 10 hr. With 20 mm. of oil these figures are only about doubled. If the oil is previously freed from oxygen by evacuation, a delay of 10 hr. may take place before the oxygen content of the water begins to rise at all. But in the case of the divers, what interests us is the escape of carbon dioxide from the interior under such conditions that *only a slight diffusion gradient* exists.

Our tests took the form of filling the divers with known gas mixtures and then following with the manometer the escape of gas into the flotation medium, the initial gas concentration of the latter being known. At the beginning of the experiment the gas inside the divers was 10 %  $\text{CO}_2$  in nitrogen, while the flotation medium was saturated with 5 %  $\text{CO}_2$  in nitrogen. In order to ensure the exactness of the internal concentration, the divers were filled in the special way to be described below (Maintenance of anaerobic conditions). The gas mixtures were prepared with the differ-

ential flowmeter of Laser (1937). The amounts of gas, in  $\lambda \times 10^{-3}$ , lost from diver to flotation medium were plotted against the time in minutes. After the loss of  $1\lambda$  exactly from the diver, the internal concentration would have dropped to 5.5 %, i.e. almost the same as that outside.

From such empirical curves the logarithmic plot of fig. 4 was derived. Here the amount of gas remaining in the diver is plotted against  $\log t$ . It will be seen that there is an initial lag, probably due to some peculiarities in the shape of the diver neck and oil drop. The important point, however, is that the straight part of the relation, if extrapolated, does not cut the

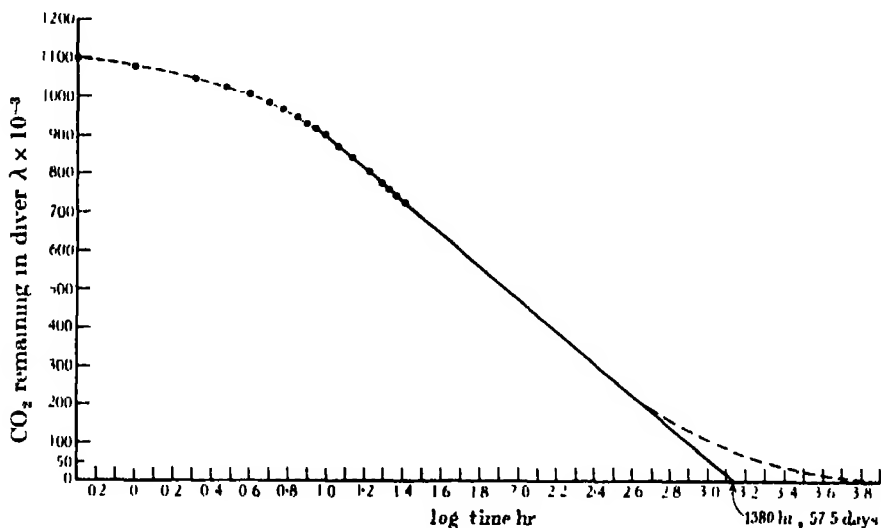


FIG. 4

base-line until after the lapse of 1380 hr., and this is undoubtedly too short a time, since the approach of the curve to the base line would almost certainly be asymptotic, and equilibrium would take even longer to attain.

If at the beginning of the experiment the concentrations of gas inside and outside the diver are the same, we have the situation that as each new increment of gas arises from any reaction going on within the diver, this will tend to leak out through the oil seal. But as the diffusion gradient is exceedingly small, it will do so exceedingly slowly. If it were necessary, an expression correcting for this loss could be worked out, allowing for the rate of gas flow through capillary tubes, etc. But we do not consider it necessary, since from fig. 4 we can calculate what the leakage per hour will be, assuming increments of different amounts within the diver, and taking



the most pessimistic assumption, namely that 1380 hr. is the time required for the whole of the extra 5% of  $\text{CO}_2$  to diffuse out into the flotation medium. The results of such a calculation show that even up to increments of  $400\lambda \times 10^{-3}$  produced within the diver, the loss does not amount to more than  $1.5\lambda \times 10^{-3}/\text{hr.}$  As the experiments with which we were concerned did not usually last beyond 3 hr. with the production of two or three hundred  $\lambda \times 10^{-3}$ , we did not feel that it was even necessary to apply the correction in practice.

Substantially the same result was obtained when 5%  $\text{CO}_2$  was inside the diver and 0% outside; we do not reproduce the curves.

In later work, to be described in a subsequent paper in this series on the application of the diver method to the determination of respiratory quotient, we had further evidence that the loss of  $\text{CO}_2$  from the divers is, in fact, inconsiderable. The  $\text{CO}_2$  was suddenly liberated from a known amount of bicarbonate solution inside the diver bulb. The solution used contained  $164.5\lambda \text{ CO}_2/\text{c.c.}$ , so that  $1.5\lambda$  in the bulb gave off  $246.5\lambda \times 10^{-3}$ , corresponding to a manometer excursion of some 10 cm. depending on the diver volume. After attainment of equilibrium, the divers were watched for some hours, but they showed no regularly perceptible tendency to fall.

#### (6) Maintenance of anaerobic conditions

In the experiments on anaerobic yeast fermentation just described, which were carried out as calibration tests, the divers were filled with gas mixture in the way recommended by the Carlsberg workers, that is to say, the gas was passed into the diver through a capillary tube for some minutes, and the oil seal then put in under a stream of the gas. The good agreement between yeast fermentation and other calibration methods showed that, for yeast at any rate, or probably any rapid chemical reaction, these precautions were sufficient. Indeed, the correspondence between successive experiments on different days with divers of different volumes was very good, thus:

Exp.	$\lambda \times 10^{-3}/\text{hr.}/1.87 \mu\text{g.}$ dry wt. yeast
39	520
41	518
42	503, 585
45	502, 570
47	520, 500, 490

But when amphibian embryo tissue was examined in the divers, an apparent negative glycolysis was commonly seen, which might amount to

as much as  $-112\lambda \times 10^{-3}$  (manometer excursion of 8.7 cm.) for a single piece of dorsal blastopore lip in 6 hr. The phenomenon persisted when pure nitrogen was substituted for  $N_2/CO_2$  mixture, so it could only be attributed to residual respiration in the presence of traces of oxygen. Under such conditions yeast, as is known, will speedily use up the remaining traces, and start its anaerobic cycle, but tissues with a weak glycolysis and accustomed to low oxygen tensions, as amphibian embryo cells undoubtedly are, will never manifest their anaerobic properties.

We were therefore under the necessity of devising some means of maintaining absolutely anaerobic conditions within the divers. We shall describe the method in its final form without mentioning problems which arose in the intermediate stages.

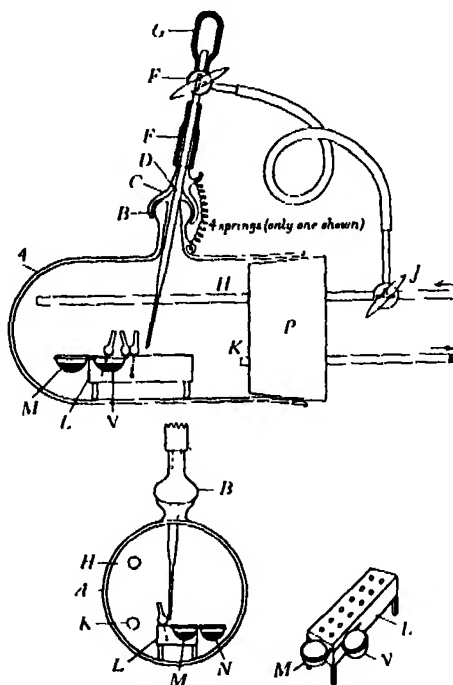


FIG. 5

Fig. 5 shows the filling chamber, *A* in the diagram. It bears a glass universal joint, *B*, through the upper shank of which (*C*), a tube *D* ending in an almost capillary point, is fixed by means of the rubber connexion, *E*. The pipette *D* is vaselined so as to be movable vertically as well as from side to side. At its upper end it carries a three-way tap, *F*, and a teat *G*. The whole chamber is closed by the rubber stopper, *P*, through which an

entry tube (*H*) brings in the gas mixture (previously purified in the usual way by passage over metallic copper in an electrically heated furnace). The entry tube also has a three-way tap, *J*, so that part of the stream can be diverted to pass through the pipette *D*. The exit tube is represented at *K*. We found it convenient to insert a wash-bottle between the electric furnace and the entry tube *H*, so as to ensure that the entering gas mixture was not unduly dry, and also a mercury trap in case of excess pressure. Inside the filling chamber there is placed a diver-carrier (*L*) constructed of a small cardboard box and match-sticks (also shown in the cross-section and in perspective). This carrier bears a wire arrangement to support two very small bowls, one for oil and one for lithium chloride (*M* and *N*). These must be in such a position that they can easily be reached by the movable pipette.

The procedure is therefore as follows: the divers, fully prepared with tissue and physiological medium, or whatever other contents are desired, are placed in their carrier, the reservoirs are filled with oil and lithium chloride, and the carrier is slid into the chamber. After the stopper has been put in, gas mixture is allowed to pass for 5 min. with a fairly rapid stream. Tap *J* is now turned so that a considerable amount of the gas mixture passes through *D*, and this is placed into each diver in turn for 2 min. By this means the divers are washed out with the gas mixture but with no danger of the entry of any air through eddy formation or otherwise. The efficacy of this gas stream can be clearly seen by the motions which it imparts to particles in the bicarbonate solution beside the tissue. When this stage is complete, taps *F* and *J* are so turned that no more gas runs through the pipette, which then takes up the desired amount of oil and delivers it into each diver neck, the movements being controlled by the teat. Finally, the pipette takes up a drop of lithium chloride solution and deposits it in the form of a ball upon the top of each diver.

In the meantime, the lithium chloride solution in which the divers are to float has been equilibrating to water-bath temperature in the vessels which have been suspended in the bath, while at the same time a stream of  $N_2/CO_2$  gas mixture has been passing through them. Both the lithium chloride and the oil are normally stored under the gas in containers which will permit of three evacuations and three fillings with the gas mixture before leaving for the night.

The efficiency of these proceedings was tested by observations of the rate of oxidation of leuco-methylene blue (Pál). If carried out exactly as described, it was found that methylene blue would remain in the reduced state for over 48 hr. without showing any sign of blue colour, but if any

one of the precautions were omitted, the presence of traces of oxygen could be detected. In particular, the lithium chloride "balls", which protect the diver contents during the few moments which elapse while the diver is being transferred from filling chamber to vessel are absolutely essential.

An experiment, in which neural folds and ventral ectoderm pieces were placed in pure nitrogen in divers filled according to the procedure just described, soon showed that no oxygen was present by remaining over a period of 7 hr. in perfect equilibrium, following precisely the slight oscillations of the thermobarometers.

#### (7) *Physiological medium*

Since amphibian embryo tissue is normally accustomed to a hypotonic environment, no Ringer solution was necessary. A very small quantity of salts, however, with bicarbonate, was found by Holtfreter (1931) to aid effectively the healing process. As will be seen by the following figures, Krebs-Henseleit Ringer solution (1932), twenty times diluted, approximates to Holtfreter solution.

	Holtfreter	Krebs-Henseleit Ringer ÷ 20
NaCl %	0.035	0.035
KCl %	0.0005	0.0018
CaCl <sub>2</sub> %	0.001	0.0028

We accordingly used this dilution, adding to it the amount of sodium bicarbonate solution normally used (concentrations given in the above paper) to bring it to equilibrium with 5% CO<sub>2</sub> in the gas mixture.

#### (8) *Dissection, introduction and removal of tissue*

Before the gastrula can be dissected it must be removed from the egg envelopes. In the Urodeles this is relatively easy, and was accomplished in the usual way with watchmaker's forceps. For Anura, however, a way was devised which may prove generally convenient. After the removal of the greater part of the jelly with scissors the gastrula was anchored to a small square piece of filter-paper by the remainder of the jelly; in this position it can be readily oriented. It is then bisected rather below the equator with a sharp pair of iridectomy scissors, after which the piece desired can easily be dissected out.

Dissections were always made with Spemann glass needles. Where special parts of the embryo were taken, mention will be made in the text. The most typical dissection, however, was that shown in fig. 6. In A the

blastopore is seen at the half-moon stage, and the dotted segment above it indicates the part called in this paper "dorsal lip of the blastopore". It includes, of course, a good deal of archenteron roof and a small amount of presumptive neural plate. Fig. 6B shows an elevation of the gastrula, C a drawing of the isolated dorsal lip region, and D a drawing of the isolated ventral ectoderm. At each side of the dorsal lip region a good deal of cleaning away of yolk endoderm cells had usually to be done, and later on the two edges of dorsal ectoderm and archenteron roof healed together leaving the appearance of a suture. Ventral ectoderm, as shown in D, generally curled up actively at the edges, but in the time elapsing between dissection and the beginning of the experiment (perhaps an hour) did not have time to heal to form a true ectodermal ball.

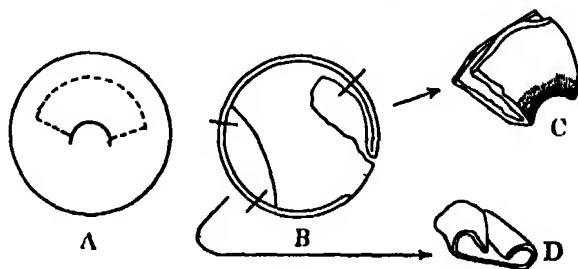


FIG. 6

In the meantime the divers had been filled\* with the Holtfreter-bicarbonate solution from the microburette. In order to put into each one the tissue isolate decided upon, the diver was completely submerged in a Petri dish containing the isolated pieces.

The next step was the removal of part of the excess Holtfreter-bicarbonate to serve as initial ammonia sample. This was accomplished by a constriction pipette of the type described by Linderström-Lang and Holter (1931, fig. 1), from which the sample was then blown out at constant pressure by compressed air into a waxed ammonia estimation tube (see below). The remainder of the unwanted Holtfreter-bicarbonate solution was now withdrawn by the microburette, so that only 2  $\lambda$  remained in the diver bulb. The diver was now ready for the anaerobic filling chamber.

At the end of the experimental manometric period, the oil seal in the neck was removed, and the diver filled up from another microburette with 0.01 N hydrochloric acid, to prevent escape of any ammonia. Then the final ammonia sample was taken off by the constriction pipette.

\* It is perhaps hardly necessary to mention that unless absolute cleanliness of the glass is maintained by a thorough use of bichromate mixture followed by prolonged washing, it will be impossible to fill the divers at all.

The problem of removing the tissue from the divers for micro-Kjeldahl analysis was solved by an unexpectedly simple procedure. The diver was inverted at the tip of a capillary test pipette bent at right angles over a Syracuse watch-glass made of paraffin wax. By exerting pressure on the teat, the tissue and the whole contents are forced out of the diver and run down the pipette dropping off at its angle into the wax receiver. From here they are taken up into another teat pipette of wider bore, which has been waxed on its inside surface, and thence blown out into the micro-Kjeldahl vessels of special pattern described in another communication (Needham and Boell 1939). Several washings of diver and waxed pipette complete the process.

#### (9) *Ammonia estimation method*

Ammonia was estimated according to the method of Linderstrøm-Lang and Holter (1933), which involves the diffusion of ammonia from a drop at the bottom of a waxed micro-test-tube\* to a hanging film at the top, followed by direct titration of the hanging film by the microburette. Magnetic stirring by small glass balls containing iron filings was used. The sample taken from the divers varied according to the constriction pipette used, from 11.89 to 15.91  $\lambda$ ; the drop of 2 N soda was 8.3  $\lambda$ , and the reception film was made with 50  $\lambda$  of distilled water, to which 7.6  $\lambda$  of 0.0283 N HCl were added from a constriction pipette. The film was back-titrated with 0.0098 N sodium borate to a colour comparison with a film of phosphate buffer solution at pH 5.9, bromocresolpurple being used as indicator.

This is perhaps the place to give details regarding the accuracy of our constriction pipettes. Of two typical examples: one delivered 7.57  $\lambda$  with an average deviation of 0.03  $\lambda$  from the mean, and a P.E. av. of  $\pm 0.0095 \lambda$ ; the other delivered 14.23  $\lambda$  with a P.E. av. of  $\pm 0.008 \lambda$ .

The microburettes used throughout the work were the very serviceable Heatley (1938) type, which substitutes a mercury bulb moving vertically for the screw syringe of Rehberg and Linderstrøm-Lang.

#### (10) *Cleaning of divers*

After use the divers are placed in strong bichromate cleaning solution, and washed before use with a great many changes of distilled water. Handling the divers is rendered very easy by the use of a capillary pipette bent at an acute angle and attached to a filter-pump. In this way, so fast can the stream be made, the diver can be washed with a million changes of

\* Hereinafter called a Holter tube.

distilled water in about half a minute. It is convenient to use a single diver over and over again, if its volume is accurately known, although another can be made if necessary in a few minutes.

(11) *General view of the apparatus*

A general diagram of the apparatus is given in fig. 7, which may be compared with a photograph (fig. 8, Plate 6).

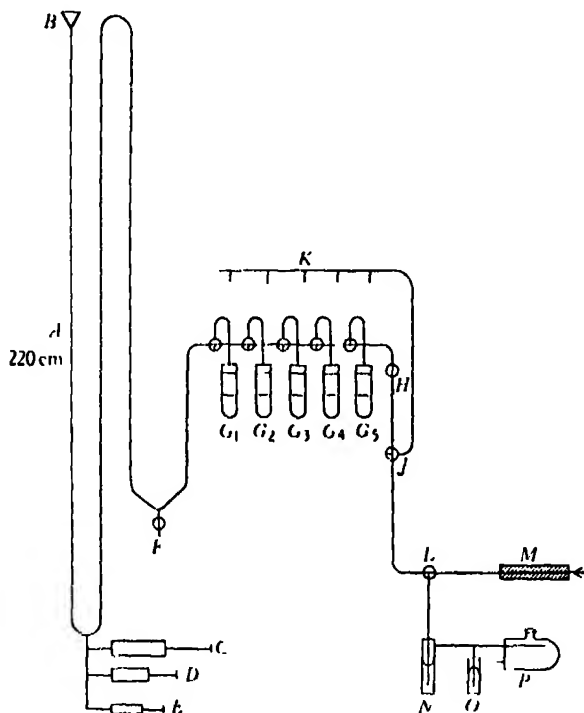


FIG. 7

Between the water manometer (220 cm. long) and the row of diver vessels is placed a tap in a convenient position (*F*, fig. 7) for adjusting the system to atmospheric pressure before beginning to take a reading. The vessels (*G*<sub>1</sub>–*G*<sub>5</sub>, fig. 7) are seen in the photograph immersed in the bath. Regulation of the bath temperature is effected by a thermionic relay system in which a current of 20  $\mu$ A passing across the thermostat junction is amplified by a wireless valve. Heating is done electrically, and as it was desired to work at or below 20° C, a cooling coil was placed at the bottom of the glass-sided bath, through which cold water from the main continually circulated. It was not found necessary in the presence of

thermobarometers to regulate the temperature more accurately than one-hundredth of a degree. Stirring was also done electrically. To prevent stray currents the bath was earthed. A magnifying glass was fixed movable along the front of the bath to facilitate the inspection of the divers at unstable equilibrium beside the zero lines. In fig. 8 the diver can just be seen resting on the bottom of the left-hand vessel.

It is undesirable that the divers should have access to the upper surface of the flotation medium, since contact with the air interface may destroy the initial state of the bubble between oil seal and lithium chloride. Small discoidal screens of silver gauze with silver wire handles were therefore placed in the vessels so as to rest upon pegs of glass rod fastened with de Khotinsky cement in such a position that the screens remained a few millimetres below the surface of the flotation medium.

(12) *Comparison of the sensitivity of the diver method  
with other micromanometric methods*

The relation of the Cartesian diver method to other micromanometric methods can best be appreciated by comparing them in the form of a table, as follows.

TABLE III

	<i>K</i> ( $\lambda$ gas change per cm. on the manometer scale)
Standard Warburg (Dixon 1934)	20
Small Warburg, as used for metabolism of tissue cultures (Laser 1932; Meier 1931)	3-7
Micromanometers of Fenn (1928), Schmitt (1933) and Duryoe (1936)	3-4
Capillary micromanometers of Gerard and Hartline (1934) and Victor (1935)	1.8-2.3
Micro-Krogh manometer (adapted from Bodine and Orr 1925)	1.6
Separate chamber micromanometer with mica mirrors optically read (Heatley, Beronblum and Chain 1938)	0.08-0.04
Very fine capillary micromanometer of Stefanelli (1937 <i>a, b, c</i> )	0.06-0.12
Cartesian diver manometer, as here described	0.008-0.022

The actual sensitivity of the diver is about one-fifth of the value given in the table; as it is easily possible to read to 2 mm. of water pressure, so it is therefore possible to measure  $0.001\lambda$ , or one-millionth part of a c.c. Normal working, however, will be in a range from ten to a hundred times as great.



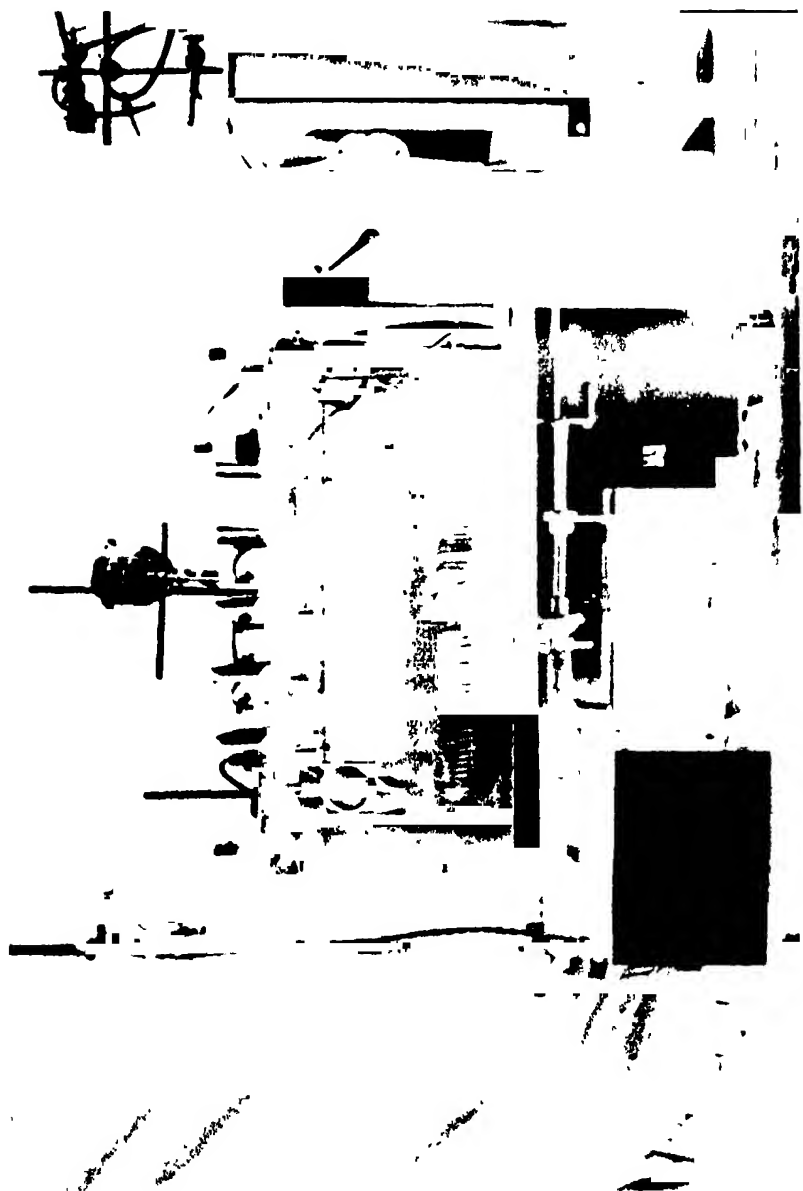


FIG. 8. General appearance of Cary sum-dye ultramicrospectrometer.



(13) *Condensed survey of the method*

It may be convenient for any who wish to apply these methods to any other or similar biological problems if at this point we give a short survey of the technique, incorporating some points not hitherto mentioned (see fig. 7).

1. The gastrulae are freed from their vitelline membranes (see § 8).
- 1a. The divers are filled with Holtfreter-bicarbonate solution and their volumes checked.
2. The regions required are dissected in Holtfreter solution.
- 2a. The density of the flotation medium is checked. The vessels are filled with it and set in the bath in their wire holders. The gas stream is passed through them, via the distributor *K*.
3. Healing is allowed to take place for 10–20 min.
4. The isolates are removed to Holtfreter-bicarbonate solution.
5. They are introduced into the divers.
6. The initial ammonia samples are removed, avoiding any injury to the tissue, and the excess Holtfreter-bicarbonate is withdrawn from each diver.
7. Initial ammonia samples are transferred to waxed Holter tubes and capped to await attention later.
- 7a. The diver necks are dried with filter-paper spills.
8. The divers are placed in their carrier, which is introduced into the anaerobic filling chamber (see § 6). Gas is led through the pipette into the divers. After 3–5 min. the oil seals, and then the lithium chloride balls, are placed in position.
9. The anaerobic filling chamber is opened and the divers are quickly transferred to their vessels.
- 9a. The silver screens are wetted with flotation medium and placed in position when each diver is in.
10. The gas stream is now so directed that it flows through the well-vaselined top of each vessel in turn, beginning with  $G_1$ , sweeping out any air contained therein. After half a minute the tap belonging to the next vessel (e.g.  $G_2$ ) is turned so that the gas stream is diverted to the next vessel. The tap of the first vessel (e.g.  $G_1$ ) is opened to the atmosphere through tap *F*, and the vessel is attached to its top and secured by springs. Finally the wire vessel-holders are removed from the bath.
11. The readings are begun, with arbitrary zeros according to the buoyancy of individual divers.
12. Manometric experimental period.
13. The vessels are taken off from their tops and out of the bath. The divers are removed (a 10 in. anatomy museum forceps is convenient), washed in water, dried, and the oil seal carefully removed with a capillary pipette attached to a filter-pump.
14. The divers are filled with dilute acid, and the final ammonia sample taken (see § 8). The ammonia estimations are proceeded with.

15. The tissue is removed and transferred to the micro-Kjeldahl vessels. After the addition of fifty  $\lambda$  of the incineration mixture, the water is driven off in an air oven at 120–130°. The rest of the method is described in Needham and Boell (1939).
16. The divers are cleaned with acid bichromate and washed with boiling distilled water.

#### (14) *Animal material*

Two sets of experiments were completed. In the first the embryos of *Rana temporaria* were used; in the second those of *Triton alpestris*.

We adopted as a routine procedure the method of pituitary gland implantations, as described by Rugh (1934, 1937) and Barth (1933). We were completely successful in obtaining and artificially fertilizing frog eggs from late September 1937 till the beginning of the natural breeding season in 1938. At the former date we used twelve frog pituitaries inserted into one of the dorsal lymph-sacs with a waxed pipette, while near the breeding season only two or three were necessary. The newt eggs were only used during their natural breeding season about the month of May.

### RESULTS

We may first take the experiments on gastrulae of *Rana*, for which the data are summarized in Tables IV and V.

It is at once evident that there is a considerable difference between the dorsal lip and ventral ectoderm. The average  $Q_{l_i}^{N_i}$  is as follows:

Dorsal lip region	...	0.63
Ventral ectoderm	...	0.21

so that the region of the gastrula where the organizer is liberated has about three times as high an anaerobic glycolytic rate as the inactive ventral ectoderm. It will be seen that in our calculations we assume that the whole of the ammonia formed during the experiment has masked an equivalent positive pressure due to lactic acid formation and its consequent carbon dioxide output (a point to which we shall return below). If, however, the ammonia production and the uncompensated  $\text{CO}_2$  production seen in the manometric readings are separately compared, the difference between dorsal lip and ventral ectoderm persists. Thus the ammonia production ( $\lambda \times 10^{-3}/\mu\text{g./5 hr.}$ ) is as follows:

Dorsal lip region	...	2.31
Ventral ectoderm	...	0.97

TABLE IV. *RANA*. DORSAL LIP

Exp. no.	<i>t</i> hr.	Dry wt. $\mu$ g.	Total glycolysis $\lambda \times 10^{-3}$	$\bar{x}_{O_2}$ $\lambda \times 10^{-3}$	Uncompensated $\text{CO}_2$ $\lambda \times 10^{-3}$ $\mu$ g./5 hr.	$\text{CO}_2$ equiv. of $\text{NH}_3$ $\lambda \times 10^{-3}$	$\text{NH}_3$ $\lambda \times 10^{-3}$ $\mu$ g./5 hr.	$\text{NH}_3$ % of total glycolysis	$Q_L^*$
8 NS 1	5	208	+ 1542	+ 112	0.54	- 1430	6.8	93	+ 1.48
2	5	264	+ 562	+ 154	0.58	- 408	1.6	72	+ 0.43
6*	5	—	+ 778	+ 133	—	- 645	—	83	—
7 NS 1	5	145	+ 900	+ 32	0.22	- 868	6.0	97	+ 1.24
2	5	222	+ 521	+ 51	0.23	- 470	2.1	90	+ 0.47
6 NS 1	3	131	+ 148	+ 108	1.37	- 40	0.5	27	+ 0.38
3	3	183	+ 389	+ 213	1.94	- 176	1.7	45	+ 0.71
6†	3	189	+ 474	+ 216	1.90	- 258	2.3	54	+ 0.84
5 NS 1	4.75	133	+ 337	+ 138	1.09	- 199	1.6	59	+ 0.54
2	4.75	54	+ 115	+ 41	0.80	- 74	1.5	64	+ 0.45
4 NS 1	5.42	144	+ 315	+ 101	0.65	- 214	1.4	68	+ 0.40
3	5.42	150	+ 314	+ 89	0.55	- 225	1.4	71	+ 0.39
3 NS 1	5	166	+ 461	+ 121	0.73	- 340	2.0	74	+ 0.56
2	5	210	+ 415	+ 150	0.72	- 265	1.3	64	+ 0.40
2 NS 1†	5	326	+ 351	+ 69	0.21	- 282	0.8	80	+ 0.21
2	5	189	+ 463	+ 199	1.05	- 264	1.4	57	+ 0.49
1 NS 1	5.16	145	+ 485	+ 68	0.46	- 417	2.8	86	+ 0.65
2	5.16	182	+ 956	+ 170	0.90	- 786	4.1	82	+ 1.02
Av.									+ 0.63
$\sigma$									32.5
P.E. av.									$\pm 0.0528$

\* Omitted from statistics.

† Notably well healed.

‡ Included a good deal of anterior ectoderm (presumptive neural plate).

TABLE V. *RANA*. VENTRAL ECTODERM

Exp. no.	<i>t</i> hr.	Dry wt. $\mu$ g.	Total glycolysis $\lambda \times 10^{-3}$	$x_{av}$ $\lambda \times 10^{-3}$	Uncompensated $CO_2$ $\lambda \times 10^{-3}$ , $\mu$ g./5 hr.	$CO_2$ equiv. of $NH_3$ $\lambda \times 10^{-3}$	$NH_3$ $\lambda \times 10^{-3}$ , $\mu$ g./5 hr. glycolysis	$NH_3$ % of total	$Q_L^*$
8 NS 3	5	—	+ 1326	+ 158	—	- 1168	—	88	—
4	5	251	+ 372	+ 60	0.24	- 312	1.2	84	+ 0.30
7 NS 3	5	153	+ 246	+ 115	0.75	- 131	0.9	53	+ 0.32
4	5	165	+ 61	+ 35	0.21	- 26	0.2	43	+ 0.07
6	5	201	+ 352	+ 68	0.34	- 284	1.4	81	+ 0.35
5 NS 4	4.75	186	+ 89	+ 19	0.11	- 70	0.4	79	+ 0.10
4 NS 2*	5.42	176	+ 167	+ 38	0.20	- 129	0.6	77	+ 0.17
4	5.42	196	+ 81	+ 38	0.18	- 43	0.2	53	+ 0.08
3 NS 3	5	186	+ 248	+ 110	0.59	- 138	0.7	56	+ 0.27
4	5	295	+ 533	+ 120	0.41	- 413	1.4	77	+ 0.36
2 NS 3	5	292	+ 191	+ 3	0.01	- 188	0.6	97	+ 0.13
4	5	367	+ 540	+ 24	0.07	- 516	1.4	96	+ 0.29
1 NS 4	5.16	133	+ 95	+ 11	—	- 84	0.7	94	+ 0.12
								Av.	+ 0.21
								$\sigma$	10.7
								P.E. av.	$\pm 0.0208$

\* Anterior ectoderm, not ectoderm from over blastocoel cavity.

giving a difference of 2.4 times in favour of the organizer region. The same is true of the uncompensated  $\text{CO}_2$  production ( $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$ ):

Dorsal lip region	...	0.82
Ventral ectoderm	...	0.28

a difference of 2.9 times in favour of the organizer region. There was moreover no difference in the ratio between uncompensated and ammonia-compensated  $\text{CO}_2$  production; thus the percentage of ammonia-compensated  $\text{CO}_2$  in the total glycolytic  $\text{CO}_2$  was as follows:

Dorsal lip region	...	70.4
Ventral ectoderm	...	73.0

This difference we do not regard as significant. Since the proportion of ammonia-compensated  $\text{CO}_2$  in the total glycolytic  $\text{CO}_2$  is so constant, a plot of ammonia productions per unit weight against  $Q_L^N$  gives a straight line.

We subjected the anaerobic glycolysis quotients given in Tables IV and V to a statistical analysis, obtaining the standard deviations and probable errors. The reliability of the difference between the two means for dorsal lip and ventral ectoderm is indicated by the ratio difference/P.E.<sub>diff.</sub> which worked out at 7.4. As this number exceeds 4.0, the difference between the dorsal lip and ventral ectoderm must be regarded as, in this case, statistically established.

One factor which undoubtedly differed a good deal as between the various experiments was the degree of healing which had taken place in the isolates before they were placed in the divers. In order to test the effect of healing, some experiments were performed in which the isolates were kept overnight for about 24 hr. in Holtfreter solution before being used. The Kjeldahl nitrogens and hence the weights were not obtained in these experiments, and the rest of the data are here omitted for lack of space, but the average uncompensated  $\text{CO}_2$  productions were so close to those usually obtained that it was felt to be unlikely that the degree of healing exerts a great influence on the anaerobic glycolysis:

	$\frac{x_{iv}}{\lambda \times 10^{-3}/5 \text{ hr.}}$
Dorsal lip region: Main series	139
Healing series	144
Ventral ectoderm: Main series	65
Healing series	73

The same conclusion was reached when the ammonia productions in  $\lambda \times 10^{-3}/\text{mg.}/5 \text{ hr.}$  within the main series were compared on the assumption

that the term "well-curling edges" in the daily descriptions of the isolates indicated better healing than when no remark was made. In the case of the dorsal lip isolates, this gave only a slight advantage to the healed pieces, and in the case of the ventral ectoderm isolates, there was a slight difference in the other direction. We conclude in general, therefore, that the degree of healing has not been a seriously disturbing factor in our experiments.

In addition to the main series of experiments on dorsal lip region and ventral ectoderm we also carried out some observations on other stages of embryonic development and on the tissues of tadpoles before metamorphosis, for comparison with adult amphibian tissue. The experiments on other embryonic stages (see Table VI) were mostly done before we had successfully surmounted the problem of maintaining absolute anaerobiosis in the divers, but the ammonia production did not seem to be greatly affected by this, as the average ammonia production in  $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$  for dorsal lip was 2.97 before the adoption of the final technique, and 2.31, as we have seen, afterwards. The following figures may therefore be regarded as fairly trustworthy. We shall assume that the ectoderm of the gastrula at the very beginning of invagination has the same ammonia production as that from over the blastocoele cavity during later stages of gastrulation. In this way we seem to see a rise of ammonia production at the beginning of the action of the primary organizer, followed by a fall:

	Ammonia production $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$
Early gastrula ectoderm	0.97
Dorsal lip region	2.97
Open neural folds	2.70
Just closed neural folds	1.30
Neural tube and somites	0.98

We should prefer to postpone for the present interpretations of these facts.

The experiments on tadpole tissues, on the other hand, were done after the adoption of the final technique. As the figures in Table VII show, the level of metabolism in tissues of tadpoles between 10 and 20 mm. length is considerably higher than that of the gastrula. While the  $Q_L^N$  for the dorsal lip of the blastopore does not often exceed 1.0, that of heart and ventral epidermis of the tadpole often exceeds 2.0. It is interesting to note that this is due to higher uncompensated  $\text{CO}_2$  production rather than to increased ammonia production. The average uncompensated  $\text{CO}_2$  output ( $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$ ), from Table VII, is 6.84, as against 0.82 for the dorsal



TABLE VI. *RANA*. OTHER EMBRYONIC STAGES

Exp. no.	<i>t</i> hr.	Dry wt. μg.	Total glycolysis $\lambda \times 10^{-3}$	$x_{NH_3}$ $\lambda \times 10^{-3}$	Uncompensated $CO_2$ $\lambda \times 10^{-3}$ /μg./5 hr.	$CO_2$ equiv. of $NH_3$ $\lambda \times 10^{-3}$	$NH_3$ $\lambda \times 10^{-3}$ /μg./5 hr. glycolysis	$NH_3$ % of total	$Q_{NH_3}^*$		
									5 hr.	1 hr.	
Open neural folds:											
i 58	1	4	284	—	—	—1005	4.4	—	—	—	
	3	4	280	—	—	—471	2.1	—	—	—	
	4	4	360	—	—	—687	2.4	—	—	—	
	5	4	290	—	—	—568	2.4	—	—	—	
Just closed neural folds:											
i 68	1	6	171	—	—	—384	1.8	—	—	—	
	2	6	162	—	—	—125	0.8	—	—	—	
Neural tube and somites:											
ii 13	1	5.25	102	+217	+58	—159	1.5	73	+0.41	—	
	2	5.25	133	+246	+190	—56	0.4	23	+0.35	—	
	3	5.25	156	+236	+170	—66	0.4	28	+0.29	—	
	4	5.25	200	+388	+294	—94	0.5	24	+0.37	—	
ii 11	1	6.5	194	—	—	—276	1.1	—	—	—	
	2	6.5	130	—	—	—244	1.5	—	—	—	

TABLE VII. *RANA*. TADPOLE TISSUES

Dry wt. μg.	Total glycolysis $\lambda \times 10^{-3}$	$\Sigma \text{CO}_2$ $\lambda \times 10^{-3}$	Uncompensated $\text{CO}_2$ $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$	$\text{CO}_2$ equiv. of $\text{NH}_3$ $\lambda \times 10^{-3}$	$\text{NH}_3$ $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr. glycolysis}$	$\text{NH}_3$ % of total	$Q_{\text{O}_2}^{\text{N}}$	
							5 hr.	1 hr.
Ventral epidermis:								
11.5 mm. tadpole	42	+ 184	+ 161	3.84	0.55	12	+ 0.88	+ 1.20
11.5 mm. tadpole	33	+ 197	+ 87	2.64	3.33	56	+ 1.19	+ 2.00
18 mm. tadpole	52	+ 101	+ 23	0.40	1.50	78	+ 0.39	+ 0.85
Heart muscle:								
11.5 mm. tadpole	36	+ 303	+ 219	6.08	2.34	28	+ 1.70	+ 1.70
11.5 mm. tadpole*	22	+ 301	+ 258	11.45	2.00	14	+ 2.70	+ 4.10
18 mm. tadpole	29	+ 470	+ 397	13.70	2.50	15	+ 3.20	+ 3.50
18 mm. tadpole*	34	+ 334	+ 334	9.80	0	0	+ 2.00	+ 2.80

\* Glucose added.

lip (Table V). The ammonia production, on the other hand, has fallen, for the average value ( $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$ ) in Table VII is 1.74, as against 2.31 for the dorsal lip (Table IV). It was noted that addition of glucose to the medium abolished the ammonia production of the 18 mm. tadpole heart muscle, but not that of the heart muscle from the younger stage. The amphibian before metamorphosis offers an inviting opportunity for the study of numerous problems in the installation of metabolic machinery, which so far seems to have been insufficiently made use of.

The reason for the increased rate of metabolism in the tadpole tissues is no doubt the disappearance of the inert yolk which is so richly contained in all the cells of the gastrula. This property of the gastrula obviated the necessity for an ultra-micro-Kjeldahl method capable of estimating accurately amounts less than  $1 \mu\text{g.}$ , for it was necessary, in order to have manageable manometric data, to take pieces containing from 3 to  $20 \mu\text{g.}$  of total nitrogen. Data for adult amphibian tissues are scarce, but the figures of +5.5 for retina (Kubowitz 1928) and +0.5 for smooth muscle (Meyerhof and Lohmann 1926) indicate that the tadpole tissues are approaching the adult level.

Throughout the foregoing discussion we have assumed that ammonia produced by the tissue during the manometric period will bind lactate molecule for molecule and so mask a glycolysis which ought to manifest itself manometrically. For mammalian tissues Dickens & Greville (1933) showed that the amounts of ammonia produced were so small as to be insignificant from this point of view, but it is clear that in the amphibian embryo we have a different state of affairs, where no less than 70 % of the total glycolysis is masked by concurrent ammonia production. Although it has always been assumed that ammonia will mask  $\text{CO}_2$  production in this way (Warburg *et al.* 1930, p. 128), we carried out a series of experiments in which, with known amounts of ammonia from ammonium carbonate, phosphate buffer at pH 7.4 was substituted for 2 N soda in the Linderström-Lang-Holter method, and we were able to assure ourselves that at any rate by far the greater part of the ammonia remains in the solution at that pH. If the relation, under the conditions of our experiments, were not quite quantitative, it would simply mean that our  $Q_N^{\text{L}}$  figures are too high by a small percentage, and since the proportion of compensated and uncompensated  $\text{CO}_2$  is the same in both dorsal lip region isolates and ventral ectoderm isolates, our conclusions would not be affected by this correction.

It is of interest to compare the figures for ammonia production obtained in the present work with those for the ammonia production of intact eggs. The paper of Białaszewicz and Mincówna (1921) on this subject stands

alone in the literature, so we made a few experiments on intact gastrulae in Thunberg tubes to confirm the conclusions of these authors. We were able to get a figure in close agreement with that of Białasiewicz and Mincówna for ammonia production in aerobic conditions, but for anaerobic conditions there was a discrepancy between direct estimations on intact eggs and estimations in the divers.

According to the Polish authors, a gastrula excretes  $1.4 \mu\text{g.}$  ammonia/24 hr., which corresponds to an excretion of  $1.17 \mu\text{g./mg.}$  dry wt. gastrula/24 hr. Our estimations gave  $1.2 \mu\text{g./mg.}$  dry wt. gastrula/24 hr. Under anaerobic conditions, however, the value was a little less, about  $1 \mu\text{g./mg.}$  dry wt. gastrula/24 hr., although from the ammonia productions observed on the isolates in the divers, a value of  $7.95 \mu\text{g./mg.}$  dry wt. gastrula/24 hr. would have been expected. We can only suggest that it is probably difficult, even with an electric vacuum pump, as used in this case, to free the egg-jellies completely from all traces of oxygen.\*

In the tables of this paper it will have been noticed that the amounts of tissue used in the divers are given in terms of dry weight. This was calculated in every case from the Kjeldahl nitrogen figure, a uniform dry weight/nitrogen ratio having previously been established. The figures for this are shown in Table VIII. A micro-balance sensitive to  $1 \mu\text{g.}$  was used. 13.7 and 15.1 were selected as the values for dorsal lip and ventral ectoderm respectively, partly because the half-moon stage is the central point in gastrulation, and partly because these figures most nearly approach what would be expected from theory. If all the solid were protein, the ratio would be 16; if half of it were fat, the ratio would be 8. From the data in the literature on the composition of amphibian eggs (Needham 1931) it can be seen that not more than 20% of the dry weight is fat. The ratio would thus be expected to be nearer 16 than 8.

It may be added that the use of Kjeldahl nitrogen values as a measure of the amount of tissue taken in calculating  $Q\dot{N}_2$  depends, of course, on the assumption that the dorsal lip and ventral ectoderm isolates contain about the same proportion of non-respiring yolk material. As it is likely that the dorsal lip region contains rather more than the ventral ectoderm, the values for the former reported in this paper are probably all slightly on the low side, and the difference between dorsal lip and ventral ectoderm therefore larger than it appears to be.

The second set of experiments were performed on embryos of the newt,

\* In addition, it must be remembered that the isolates used are not taken from the yolk-laden regions of the egg, and would hence be expected to show a higher activity.

*Triton alpestris*, which, although the classical material of experimental embryology, did not prove nearly so satisfactory for the present purpose as the anuran gastrulae. Even when the healing was perfect, so much mucus was secreted by the isolates that it was hard to get them into the divers absolutely uninjured, and they also showed a much greater tendency to go to pieces during the manometric period, possibly owing to their inferior resistance to cytolysis at air-water interfaces. Unlike the artificially fertilized eggs of the frog, the newt eggs were fairly often bad, and it was not easy to have full confidence that a gastrula, even though picked with care, would have continued normal development had it been allowed to do so.

TABLE VIII. DRY WEIGHT/NITROGEN RATIO. *RANA TEMPORARIA*

Numbers of isolated pieces taken varied from 15 to 30.

Exp. no.	Description	Dry wt. mg.	Total N mg.	N % dry wt.	Dry wt./N ratio
1 60	Dorsal lip, $\frac{1}{4}$ -moon yolk-plug	2.842	0.214	7.52	13.3
	Ventral ectoderm, $\frac{1}{4}$ -moon yolk-plug	4.724	0.250	5.30	18.8
	Dorsal lip, $\frac{1}{4}$ -moon yolk-plug	8.999	0.654	7.28	13.7
	Ventral ectoderm, $\frac{1}{4}$ -moon yolk-plug	6.120	0.405	6.63	15.1
11 45	Dorsal lip, $\frac{1}{4}$ -moon yolk-plug	1.921	0.111	5.80	17.3
	Ventral ectoderm, full-moon yolk-plug	2.228	0.121	5.45	18.4
11 161	Blastula roof	2.135	0.128	6.00	16.7

In spite of these difficulties, however, a series of some twenty experiments was run, giving the following differences, all in favour of the dorsal lip region.

	Dorsal lip region	Ventral ectoderm
$Q_2^*$	0.23	0.14
Ammonia production $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$	0.75	0.50
Uncompensated $\text{CO}_2$ $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$	0.30	0.22

These experiments, therefore, so far as they go, confirm in general trend the results obtained on the anuran tissue.

In connexion with the differential behaviour of the regions of the gastrula, it may be remembered that according to Bellamy (1919) and Bellamy and Child (1924) disintegration begins in the dorsal lip of the blastopore when embryos are treated with toxic concentrations of mercuric chloride, potassium cyanide, and ammonia. The claim was contested by Cannon (1923) and the original papers must be referred to by those who wish to estimate its probability.

More recently Fischer and Hartwig (1936) and Piepho (1938), using various vital dyes, have found that when the stained gastrulae are placed in *strictly anaerobic conditions*, there is a more rapid reduction of the dye in the dorsal lip region than elsewhere. It is hard at the present time to be precise regarding the significance of these findings, though they must surely indicate greater activity of oxido-reduction systems in the organization centre relative to the other parts of the embryo.

Lastly Brachet (1938) has been able to show with the nitroprusside test on the unpigmented gastrulae of *Triton cristatus* a local accumulation of fixed —SH groups in the dorsal lip of the blastopore.

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#### SUMMARY

1. The principle of the Cartesian diver, employed by Linderstrøm-Lang for the purpose of an ultramicromanometer, gives an instrument 1500 times more sensitive than the Warburg manometer. The delicacy of the method can be gauged by the fact that whereas 1 cm. on the Warburg manometer scale corresponds to a gas change of about 20 cu.mm., 1 cm. on the diver manometer scale corresponds to a gas change of about 0.008 cu.mm. The method of calibration of the diver manometer has been fully investigated and is described in the text.

2. The use of the diver manometer for measuring the anaerobic glycolysis of pieces of amphibian embryo tissue of about 100  $\mu$ g. dry weight necessitated several new and special techniques, such as those for the introduction and removal of the tissue, the maintenance of strictly anaerobic conditions, the use of a new flotation medium (lithium chloride), ammonia and Kjeldahl estimations, etc. All these procedures are described in the text.

3. The dorsal blastopore lip (organization centre) region of the amphibian gastrula has a higher anaerobic glycolysis and a higher anaerobic ammonia production than the ventral ectoderm. The difference, established by statistical test, is of the order of three times.

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## Morphogenesis and metabolism: studies with the Cartesian diver ultramicromanometer

### II. Effect of dinitro-*o*-cresol on the anaerobic glycolysis of the regions of the amphibian gastrula

BY E. J. BOELL AND JOSEPH NEEDHAM

*The Biochemical Laboratory, Cambridge*

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#### INTRODUCTION

In the preceding paper of this series a considerable difference in anaerobic glycolytic rate was demonstrated to exist between the dorsal lip of the blastopore in the amphibian gastrula and the ventral ectoderm. It was therefore of some interest to see whether this difference could be diminished or even abolished by any artificial treatment. In recent years there has been a wide extension of our knowledge of chemical substances which inhibit and stimulate the respiration and glycolysis of isolated tissues, but for respiration, which can be accelerated by the addition of many oxidizable



and reducible dyes to act as accessory hydrogen transporters in the cell, the progress has gone further than for glycolysis. However, the initial discovery of Heymans and Bouckaert (1928) that nitrophenol derivatives have a profound effect on animal metabolism has led to much study of these substances. Our experiments originated from the fact reported by Dodds and Greville (1934) that dinitro-*o*-cresol stimulates both aerobic glycolysis and respiration in mammalian tissues.

### METHODS

The general technique employed was exactly the same in all particulars as that of the preceding paper, with the exception that the Holtfreter-bicarbonate solution used contained 4, 6-dinitro-*o*-cresol in a concentration of  $1 \times 10^{-5}$  M, which was shown by Bodine and Boell (1938) to be near the maximally effective concentration in the case of grasshopper embryos. Gastrulae of *Rana temporaria* were obtained and prepared for the experiments following the previously described technique. The isolated pieces were in the nitrocresol solution for approximately 40 min. before the manometric readings began; this was the time taken to remove the initial ammonia samples and the excess Holtfreter-bicarbonate solution, and to prepare the divers anaerobically in the filling chamber.

### RESULTS

The results are incorporated in Table I which should be compared with Tables IV and V of the preceding paper.

From the averages assembled in Table II it can be seen that the effect of dinitro-*o*-cresol is to diminish considerably the difference between the dorsal lip region and the ventral ectoderm by raising the metabolic level of the latter rather than the former. But at the same time it is to be noticed that this effect is much more pronounced upon the uncompensated  $\text{CO}_2$  than it is on the ammonia production (and hence the compensated  $\text{CO}_2$ ). Consequently the  $Q_L^{\text{N}}$ , which is the balance between these two  $\text{CO}_2$  productions, is affected only to an intermediate degree. There is a rise of nearly 300 % in the uncompensated  $\text{CO}_2$  production of the ventral ectoderm, while the corresponding effect on dorsal lip region is only 35 %. On the other hand the dorsal lip region's ammonia production is apparently not affected at all by dinitrocresol, while that of the ventral ectoderm only increases by 67 %. The overall result on the  $Q_L^{\text{N}}$  is therefore a very slight increase, only 16 %, for the dorsal lip, and a considerable increase, 157 %, for the

TABLE I. EFFECT OF DINITRO-O-CRESOL ON METABOLISM OF REGIONS OF *RANA TEMPORARIA* GASTRULAE

Exp. no.	t hr.	Dry wt. μg.	Total glycolysis $\lambda \times 10^{-3}$	$x_{CO_2}$ $\lambda \times 10^{-3}$	Uncompensated CO <sub>2</sub> $\lambda \times 10^{-3}/\mu g./5$ hr.	CO <sub>2</sub> equiv. of NH <sub>3</sub> $\lambda \times 10^{-3}$	NH <sub>3</sub> $\lambda \times 10^{-3}/\mu g./5$ hr. glycolysis	NH <sub>3</sub> % of total	Q <sub>10</sub>
Dorsal lip region:									
1 DNC	1	182	—	—	—	— 350	2.40	—	—
6	4	120	+ 892	+ 237	2.47	— 655	6.82	73	1.86
2 DNC	6	247	+ 1242	+ 132	0.54	— 1110	4.50	89	1.01
3 DNC	1	218	—	+ 212	1.08	—	—	—	—
1a	4.5	147	—	+ 91	0.69	—	—	—	—
6	4.5	165	—	+ 218	1.48	—	—	—	—
4 DNC	1	312	+ 428	+ 242	0.86	— 186	0.67	44	0.31
1a	4.5	271	+ 283	+ 166	0.68	— 117	0.48	41	0.23
6	4.5	193	+ 324	+ 121	0.70	— 203	1.18	63	0.37
5 DNC	1	175	+ 466	+ 220	1.39	— 246	1.57	53	0.59
1a	4.5	251	—	—	—	— 43	0.19	—	—
6	4.5	257	—	+ 277	1.19	—	—	—	—
Ventral ectoderm:									
1 DNC	3	144	+ 416	+ 144	1.25	— 272	2.36	65	0.72
2 DNC	3	248	—	+ 197	0.79	—	—	—	—
3 DNC	3	281	—	+ 252	1.00	—	—	—	—
3a	4.5	—	—	+ 287	—	—	—	—	—
4 DNC	3	—	+ 973	+ 306	—	— 667	—	69	—
3a	4.5	169	+ 278	+ 174	1.14	— 104	0.69	37	0.37
5 DNC	3	322	+ 778	+ 333	1.15	— 445	1.54	57	0.54
3a	4.5	—	+ 477	+ 264	—	— 213	—	45	—

TABLE II. CHANGES PRODUCED IN THE METABOLISM OF AMPHIBIAN GASTRULA REGIONS BY TREATMENT WITH DINITRO-*o*-CRESOL

$Q_L^N$	Dorsal lip region	Ventral ectoderm
Normal	0.63	0.21
Dinitro- <i>o</i> -cresol	0.73	0.54
Difference	+0.10	+0.33
% rise	16	157
Uncompensated $\text{CO}_2$ ( $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$ )		
Normal	0.82	0.28
Dinitro- <i>o</i> -cresol	1.11	1.07
Difference	+0.29	+0.79
% rise	35	282
Ammonia production ( $\lambda \times 10^{-3}/\mu\text{g.}/5 \text{ hr.}$ )		
Normal	2.31	0.97
Dinitro- <i>o</i> -cresol	2.23	1.62
Difference	-0.08	+0.65
% change	-3	+67

ventral ectoderm. This increase does not suffice to abolish altogether the difference between the two regions, but instead of being three times it is reduced to very much under twice.

### DISCUSSION

The action of dinitro-*o*-cresol in stimulating the metabolism of the relatively inactive ventral ectoderm (presumptive epidermis) rather than that of the relatively active dorsal lip region (presumptive chordamesoderm) is very suggestive. In particular it reminds us of two other cases in which the lower metabolic level is preferentially stimulated, namely the grasshopper embryo during diapause and during active development, and the sea-urchin egg before and after fertilization. Both these, however, are cases of aerobic, not anaerobic metabolism.

The former has been studied by Bodine and Boell (1938) as the latest of a series of investigations on the developing orthopteran embryo, details of which will be found in this publication. As is well known, the grasshopper embryo develops actively at first for a few weeks but then enters a state of dormancy, the diapause, in which no morphological development occurs. This lasts several months, after which a period of intense activity, both morphological and metabolic, leads in three weeks to the end of

embryonic development. Respiratory rate is considerable during the two active periods, but sinks to a low steady state during the diapause. Since the respiration in the two active periods is powerfully inhibited by KCN and CO, while that of the diapause is not, it is to be supposed that diapause respiration proceeds mainly by "non-ferrous", possibly flavin-catalysed, systems. It was now found that taking normal respiration as 100, the diapause respiration was raised by nitrophenols to 375, while that of the active periods only to 250. The extra respiration was cyanide-sensitive. The fact that the respiratory quotient was raised from the usual diapause level of 0.75 to unity was at first attributed to the extra respiration being at the expense of carbohydrate, but it was not sensitive to fluoride or iodoacetate, and ammonia estimations showed that at least three-quarters of it was due to protein breakdown.

In the case of the amphibian gastrula, however, the fact that dinitro-o-cresol did not raise the ammonia production to any extent suggests that its action is mainly if not entirely upon mechanisms of carbohydrate utilization. This agrees with the findings of Ronzoni & Ehrenfest (1936) on mammalian muscle.

The parallel with the sea-urchin egg is of much the same kind. A considerable literature exists on the respiratory mechanism here involved, for which the paper of Korr (1937) may be consulted, but it is sure from the work of Runnström (1930) and others that the respiration of the unfertilized egg is not sensitive to cyanide, while that of the fertilized egg, proceeding at a higher rate, is so. Later, Runnström (1935) found that although pyocyanin, added as an accessory hydrogen transporter, increased the respiratory rate of both unfertilized and fertilized eggs, its effect was of the order of 200 % on the former and only 80 % on the latter. The effects of nitrophenols on the sea-urchin embryo have been studied by Clowes and his collaborators (see Krahl and Clowes 1938, for the literature). Most of their attention was devoted to the interesting fact, discovered by them, that concentrations of nitrophenols and halophenols which give the maximal stimulation to respiration also cause a reversible blockage in the mechanism of cell division. In one place, however (Clowes and Krahl 1934), they state that the effect of nitrophenols on respiration was greater for the unfertilized than for the fertilized eggs—a six times increase against a four times increase. Thus while the respiration of the fertilized egg is normally four times that of the unfertilized egg, after both had been treated with dinitrophenol, it was only two and a half times as great.

An entirely different type of reagent, dimethyl-*p*-phenylene-diamine, was found by Runnström (1932) to raise both unfertilized and fertilized

egg respiration to the same high level, indicating that the low activity of the unfertilized egg is not due to any deficiency of indophenol oxidase.

All these phenomena resemble to some extent the state of affairs found for the amphibian gastrula regions. In general we may say that wide variations in metabolic activity exist in correlation with various physiological and morphogenetic states. The regions or states of high activity may or may not be in a condition of maximum possible activity, but the regions or states of low activity are certainly damped down in some way or other. For respiration, this damped state may be due to a block in the chain of carriers in the Warburg-Keilin system, so that such respiratory activity as there is has to proceed by way of a "non-ferrous" system. For glycolysis we can as yet form little idea of the damping mechanism. It may be that the theories of the Pasteur reaction, especially those which postulate that the glycolytic enzymes are themselves sensitive to oxidation or to other factors (see the review of Dixon 1937), will enable us to understand these damped states, and the connexion which they have with the concurrent physiological and morphogenetic events. In the case of the amphibian gastrula, at any rate, the fact that the organizer substance, or evocator, is liberated in a region of high activity, and not in a region physiologically damped, can hardly be without significance.

If the liberation of the evocator were coupled in any simple way with the intensified glycolytic mechanisms in the dorsal blastopore lip, it might be expected that dinitro-*o*-cresol would bring about a liberation of the evocator in pieces of normal ventral ectoderm isolated and exposed to its influence. The paper of Waddington, Needham and Brachet (1936) reported a few experiments of this kind, but unlike the results with methylene blue, no positive effects were obtained. This may have been due to the relatively small number of isolations made, and the subject deserves further examination. Subjecting intact frog embryos to the action of 2, 4-dinitrophenol during early developmental stages gave Dawson (1938) nothing but a general retardation and some persistent yolk-plugs, while Finkelstein and Schapiro (1937), who implanted the same substance dispersed in agar into the blastocoele cavity, could obtain no positive neural inductions.

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## SUMMARY

1. The effect of dinitro-*o*-cresol on the anaerobic glycolytic rate and ammonia production of the dorsal lip region and ventral ectoderm of the frog gastrula was investigated with the methods described in the preceding paper of the series.

2. The greatest effect was an increase of some 300 % in the uncompensated CO<sub>2</sub> output of the ventral ectoderm. Ammonia production was much less affected. Anaerobic glycolytic rate was therefore increased to an intermediate extent. All the changes were much more marked in the ventral ectoderm than in the dorsal lip region.

3. Attention is drawn to a certain parallelism between the state of affairs in the amphibian gastrula and that in the grasshopper embryo during diapause and the sea-urchin egg before and after fertilization.

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# Morphogenesis and metabolism: studies with the Cartesian diver ultramicromanometer

## III. Respiratory rate of the regions of the amphibian gastrula

BY E. J. BOELL AND JOSEPH NEEDHAM

*The Biochemical Laboratory, Cambridge*

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### INTRODUCTION

For a survey of the metabolic properties of the regions of the amphibian gastrula a knowledge of the relative respiratory rates is indispensable. This had been the aim of a number of previous investigations; to these we shall refer in the Discussion.

An account of the reasons why a study of the metabolic properties of the regions of the amphibian gastrula is of importance has already been given in the introduction to the first paper of this series.

### METHODS AND MATERIAL

The divers used for experiments on oxygen consumption were of the same dimensions as those already described in the paper on the measurement of anaerobic glycolysis (Boell, Needham and Rogers 1938), but were arranged in a different way. As fig. 1 shows, the neck of the diver was coated with a uniform layer of high-melting-point paraffin wax. This does not change the behaviour of the lithium chloride meniscus nor of the oil seal, but permits of the placing of a drop of 0.5 N soda at the bottom of the neck just above the bulb. The menisci of this drop are nearly straight, as shown in the diagram, and it acts as a very efficient absorbent of CO<sub>2</sub>. The drop of soda, about 2λ in volume, is allowed for in the calibration equation by the insertion of a special term.

Holtfreter solution without bicarbonate was used as physiological medium, and the whole process of preparing the divers was done in air. The introduction of the tissue into the divers and the subsequent removal of the excess Holtfreter solution was carried out as described in the first paper of the series. Special tests with indicators showed that there was no danger whatever of the alkali drop leaving the neck and coming down into the bulb.

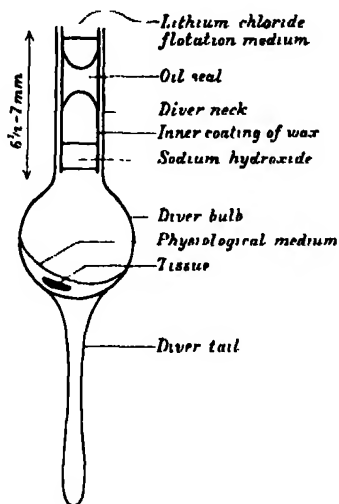


FIG. 1

Being desirous of making a test with the apparatus on some biological object previously well studied, we made a few experiments on diapause embryos of the grasshopper *Melanoplus differentialis*. In 3 hr. several of these took up an average of  $73.6\lambda \times 10^{-3} \text{ O}_2/\text{hr.}$  each or just about  $0.074 \text{ cu.mm./hr./embryo}$ . The usual range is from 0.05 to 0.08.

The dissection of the pieces of the gastrulae was accomplished in the usual way with Spemann glass needles. Embryos of the African toad, *Discoglossus pictus*, and the axolotl, *Amblystoma mexicanum*, were used. For reasons to be mentioned in the Discussion, it seemed desirable to use as far as possible pieces of dorsal lip region and ventral ectoderm taken from the same embryo, and this was almost always done. It was also thought necessary to pay particular attention to the stage of development, and for this purpose the morphological normal table of Harrison (unpub.) was used. Unfortunately, none of the existing normal tables for the amphibia include a sufficiently large number of stages during gastrulation, so we inserted some intermediate stages, as indicated in Table I.



TABLE I

Stage	Stages of Harrison (unpub.) for <i>Amblystoma</i> <i>punctatum</i>	Stages of Knight (1938) for <i>Triton</i> <i>alpestris</i>	Stages of Pollister and Moore (1937) for <i>Rana</i> <i>sylvatica</i>
Blastula	8	—	—
Late blastula	9	10	9
Blastopore just visible	10	11	10
$\frac{1}{4}$ -moon yolk-plug, blastopore "sickle-shaped"	10 $\frac{1}{2}$	11 $\frac{1}{2}$	—
$\frac{1}{4}$ -moon yolk-plug	10 $\frac{1}{2}$	12	11
$\frac{3}{4}$ -moon yolk-plug, blastopore "horseshoe-shaped"	11	13	—
Large full-moon yolk-plug	11 $\frac{1}{2}$	13 $\frac{1}{2}$	—
Small full-moon yolk-plug	12	14	12
First appearance of neural folds	13	16	—
Closing neural folds	18	18	14

Abbreviations used in the succeeding tables: brf, blastula roof; dl, dorsal lip of blastopore region; ve, ventral ectoderm; enf, closing neural folds.

## RESULTS

The figures for the oxygen consumption of isolated pieces of *Discoglossus* and *Amblystoma* gastrulae will be found in Tables II and III. It will be noticed that the diver method measures with ease gas intakes of the order of 0.03 cu.mm./hr. In 3 hr., the average gas change in this work amounts to about one-tenth of 1 cu.mm. We did not think it necessary to calculate  $Q_{O_2}$  in every case, but related the oxygen consumption instead to the quantity of total nitrogen found. This we express as  $Q'_{O_2}$ . It may be taken as a sufficient basis of comparison between the different regions.

Taking first the *Discoglossus* series, we find that the averages are as follows:

	$Q'_{O_2}$
Dorsal lip region	4.80
Ventral ectoderm	4.93
Closing neural folds	3.02

Assuming the dry weight/nitrogen ratios obtained in the first paper of this series, these values are equivalent to

	$Q_{O_2}$
Dorsal lip region	0.35
Ventral ectoderm	0.33

These are of the same order as the  $Q_L^N$  values previously obtained, namely 0.63 for dorsal lip and 0.21 for ventral ectoderm. But it is at once clear that unlike the results for anaerobic glycolysis, there is here no difference whatever between the metabolic activity of the two regions. Reference to Table II will show that there are three points lying outside the main zone. If we exclude them by omitting from the calculation all points above 9.0, the result is not appreciably altered (dorsal lip 4.2, ventral ectoderm 3.75). With so small a difference, a statistical analysis seemed unnecessary.

TABLE II. RESPIRATORY RATE OF REGIONS OF *DISCOGLOSSUS* GASTRULA

Exp. no.	Tissue	Harrison stage	t hr.	$\mu\text{g.}$ protein N	$\text{O}_2$ consumption		$Q'_{\text{O}_2}$	$Q_{\text{O}_2}$	
					$\lambda \times 10^{-3}$	$\lambda \times 10^{-3}/3 \text{ hr.}$			
O0	1	cnf	18	4.18	13.95	-121	-87	2.1	0.16
	2	cnf	18	4.16	22.9	-318	-230	3.4	0.26
	3	cnf	18	4.16	18.2	-151	-109	2.0	0.15
	4	cnf	18	4.16	12.35	-232	-168	4.6	0.35
O1	1	dl	12	4	8.7	-187	-140	5.35	0.39
	2	dl	12	4	6.6	-302	-225	11.4	0.87
	3	ve	12	4	5.05	-102	-76	5.0	0.33
	4	ve	12	4	2.6	-36	-27	3.5	0.23
O2	1	dl	11	4	14.5	-93	-70	1.7	0.12
	2	dl	11	4	14.5	-165	-124	2.9	0.21
	3	ve	11	4	5.1	-34	-26	2.9	0.19
	4	ve	11	4	4.8	-47	-35	2.7	0.18
O3	1	dl	$10\frac{1}{2}$	4	8.8	-67	-50	1.9	0.14
	2	vo	$10\frac{1}{2}$	4	5.4	-120	-90	5.6	0.37
	3	dl	$10\frac{1}{2}$	4	12.6	-197	-148	4.0	0.29
	4	vo	$10\frac{1}{2}$	4	8.75	-94	-71	2.7	0.18
O4	1	dl	$10\frac{1}{2}$	2	9.2	-82	-123	4.5	0.33
	2	ve	$10\frac{1}{2}$	2	3.9	-77	-116	9.8	0.65
	3	dl	$10\frac{1}{2}$	2	5.15	-72	-108	7.0	0.51
	4	vo	$10\frac{1}{2}$	2	3.4	-83	-125	12.1	0.80
O5	1	dl	12	3	12.8	-109	-109	2.9	0.21
	2	vo	12	3	5.0	-66	-66	4.4	0.29
	3	dl	12	3	5.9	-95	-95	5.4	0.39
	4	vo	12	3	6.8	-69	-69	3.3	0.22
O6	1	vo	11	3.5	8.9	-136	-117	4.4	0.29
	2	dl	11	4.5	11.5	-260	-173	5.1	0.37
	3	vo	12	3.5	10.2	-106	-91	3.0	0.20
	4	dl	12	4.5	8.7	-215	-142	5.5	0.40

Abbreviations and stage numbers as in Table I. Brackets indicate that both pieces came from the same gastrula.

TABLE III. RESPIRATORY RATE OF REGIONS OF *AMBLYSTOMA* GASTRULA

Exp. no.	Tissue	Harrison stage	t hr.	$\mu\text{g.}$ protein N	O <sub>2</sub> consumption		Q'_{O_2}
					$\lambda \times 10^{-4}$	$\lambda \times 10^{-3}/\mu\text{g.}/3 \text{ hr.}$	
11 O1	1 brf	8½	3.25	18.0	-159	-147	2.7
	2 brf	8½	3.25	14.7	-98	-91	2.1
	3 brf	8½	3.25	16.6	-147	-136	2.7
	4 brf	8½	3.25	22.0	-137	-127	1.9
11 O2	1 brf	9	3	12.0	-112	-112	3.1
	2 brf	9	3	8.7	-126	-126	4.8
	3 brf	9	3	15.6	-118	-118	2.5
	4 brf	9	3	20.6	-202	-202	3.3
11 O3	1 ve	10	3	14.6	-118	-118	2.7
	2 dl	10	3	11.3	-54	-54	1.6
	3 ve	10	3	14.1	-92	-92	2.2
	4 dl	10	3	18.4	-120	-120	2.2
11 O4	1 ve	10	3.25	13.2	-106	-98	2.5
	2 dl	10	3.25	—	-133	-123	—
	3 ve	10	3.25	5.9	-115	-106	6.0
	4 dl	10	3.25	11.5	-79	-73	2.1
11 O5	1 dl	10½	3.5	—	-171	-147	—
	2 ve	10½	3.5	8.9	-113	-96	3.6
	3 dl	10½	3.5	—	-145	-125	—
	4 ve	10½	3.5	9.1	-87	-75	2.7
11 O6	1 dl	10½	3	14.6	-131	-131	3.0
	2 ve	10½	3	17.9	-86	-86	3.6
	3 dl	10½	3	12.7	-113	-113	3.0
	4 ve	10½	3	2.8	-46	-46	5.5
11 O7	1 dl	10½	3.67	4.9	-173	-141	9.6
	2 ve	10½	3.67	6.25	-93	-76	4.1
	3 dl	10½	3.67	9.8	-198	-162	5.5
	4 ve	10½	3.67	13.1	-88	-72	1.8
11 O8	1 dl	10½	3.5	18.4	-151	-129	2.3
	2 ve	10½	3.5	12.2	-90	-77	2.1
	3 dl	10½	3.5	18.3	-179	-154	2.8
	4 ve	10½	3.5	14.7	-88	-76	1.7
11 O9	1 dl	10½	3	23.0	-118	-118	1.7
	2 ve	10½	3	18.8	-94	-94	1.7
	3 dl	11	3	14.8	-120	-120	2.7
	4 ve	11	3	5.3	-134	-134	8.4
11 O10	1 dl	11	3	16.3	-111	-111	2.3
	2 ve	11	3	11.7	-76	-76	2.3
	3 dl	11	3	8.3	-136	-136	5.5
	4 ve	11	3	9.0	-48	-48	1.8

Abbreviations and stage numbers as in Table I. Brackets indicate that both pieces came from the same gastrula.

TABLE III (continued)

Exp. no.	Tissue	Harrison stage	t hr.	$\mu\text{g.}$ protein N	$\text{O}_2$ consumption		$Q'_{\text{O}_2}$
					$\lambda \times 10^{-3}$	$\lambda \times 10^{-3}/\mu\text{g.}/3 \text{ hr.}$	
11 O11	1 dl	11	2	12.5	- 51	- 77	2.0
	2 ve	11	2	7.1	- 47	- 71	3.3
	3 dl	11	2	16.0	- 78	- 117	2.4
	4 ve	11	2	6.8	- 49	- 74	3.6
11 O12	1 dl	10½	3.08	8.8	- 95	- 93	3.5
	2 ve	10½	3.08	4.1	- 36	- 35	2.9
	3 dl	10½	3.08	8.9	- 107	- 104	3.9
	4 ve	10½	3.08	7.1	- 56	- 55	2.6
11 O13	1 dl	10½	3.16	14.8	- 121	- 115	2.6
	2 ve	10½	3.16	9.1	- 99	- 94	3.4
	3 dl	10½	3.16	16.5	- 120	- 114	2.3
	4 ve	10½	3.16	12.3	- 64	- 61	1.6
11 O14*	1 dl	10½	3	18.7	- 210	- 210	3.7
	2 ve	10½	3	11.1	- 87	- 87	2.6
	3 dl	10½	3	20.1	- 106	- 196	3.3
	4 ve	10½	3	—	- 135	- 135	—

Abbreviations and stage numbers as in Table I. Brackets indicate that both pieces came from the same gastrula.

\* In the presence of  $1 \times 10^{-5} \text{ M}$  4:6-dinitro-*o*-cresol.

Exactly the same conclusion was reached for the *Amblystoma* series. Averages are as follows:

	$Q'_{\text{O}_2}$
Blastula roof	2.63
Dorsal lip region	3.21
Ventral ectoderm	3.18

These figures give  $Q_{\text{O}_2}$  values of the order of 0.22. Omitting the two high points has no effect. It will be seen from Table III that an orientation experiment was made with dinitro-*o*-cresol. It had apparently no effect on the respiratory rate. The *Amblystoma* series is the more reliable of the two, as the axolotl gastrula tissue seemed more resistant to the experimental conditions.

In order to make sure that oxygen diffusion was not a limiting factor, we plotted all the points obtained on a graph relating micrograms of protein N to  $-x_{\text{O}_2}$  for 3 hr. There was no tendency for unduly small oxygen-uptakes at the higher end of the tissue scale; all the points lay in a rather wide scatter (due no doubt to varying amounts of yolk, endoderm cells, etc.) around a line at  $45^\circ$ .

## DISCUSSION

A good deal of work has been done on the respiration of intact amphibian gastrulae, so it was of interest to compare the figures in the literature with those found by the diver method on isolated regions. The following table (Table IV) summarizes them.

TABLE IV

	$T^{\circ}$	$\lambda$ O <sub>2</sub> uptake/100 embryos/hr.	$\lambda$ CO <sub>2</sub> output/100 embryos/hr.
Bialasiewicz and Błędowski (1915):			
<i>Rana temporaria</i> , gastrulae	20	24.5	—
Saunders (1923):			
<i>Rana temporaria</i> , gastrula	14	—	24.4
Parnas and Krasinska (1921):			
<i>Rana temporaria</i> , gastrulae	15	13.7-25.0	—
Wills (1936):			
<i>Amblystoma punctatum</i> , Harrison stage 10, gastrulae	25	34	—
<i>Triturus torosus</i> , Harrison stage 10, gastrulae	25	39	—
<i>Rana pipiens</i> , Harrison stage 10, gastrulae	25	39	—
Stofanelli (1937):			
<i>Rana fusca</i> , cleavage stages	?	(9-18) av. 13	—
Brachet and Shapiro (1937):			
<i>Rana sylvatica</i> , gastrulae:	25	—	—
Dorsal lip hemisphere	—	70.6	—
Ventral ectoderm hemisphere	—	48.1	—
Brachet (1934a):			
<i>Rana temporaria</i> , gastrulae	?	29.9	—
Atlas (1938):			
<i>Rana pipiens</i> , gastrulae	15	12	—

If now we compare these results with those here reported, we find that the respiration of dorsal lip region and ventral ectoderm is a good deal higher than would be expected from the volume of embryo occupied by them. From our data on the axolotl gastrula, the average amount of protein N taken was 11.1  $\mu$ g. or 1/32nd part of the total protein nitrogen in the embryo. As  $Q'_{O_2}$  is of the order of 3, the oxygen consumption for the whole embryo would be 106 $\lambda$ /100 embryos/hr., a figure considerably higher than any given in the table. It should, however, be remarked that in Brachet and Shapiro's measurements, the value of 70 $\lambda$ /100 embryos/hr. was also much above the average for the whole intact embryo. We can conclude certainly that the yolk endoderm must have an extremely low respiratory rate and occupies a large proportion of the total weight and nitrogen of the embryo.

We have now to consider how the present results contrast with those previously reported in the literature for the two regions. Let us briefly recapitulate the progress made hitherto. On the whole, the first set of experiments made by Brachet (1934*b*) may be disregarded since the amount of material in isolated pieces was not quantitatively determined in any way. In the second set, however (Brachet 1936), a micro-Kjeldahl method was used, and although no detailed figures were given, the ratio of CO<sub>2</sub> production dorsal lip/ventral ectoderm was 1.84/1.00, determinations being made both by an indicator-change method and by a modification of the Meyerhof-Schmidt respiratory quotient method. By this latter method, the ratio of O<sub>2</sub> consumption dorsal lip/ventral ectoderm was 1.37/1.00. About the same time Waddington (in Waddington, Needham and Brachet 1936) measured the oxygen uptake of isolated pieces in a modified Gerard-Hartline capillary micromanometer, the dry weight being obtained by weighings on a microbalance. In this case, however (*Triton alpestris*), the respiratory rate turned out to be identical as between dorsal lip region and ventral ectoderm:

	$Q_{O_2}$
Dorsal lip region	0.227
Ventral ectoderm	0.212

figures which agree very closely with those reported in the present paper. Finally, Brachet & Shapiro (1937) devised an ingenious apparatus in which the intact gastrula was located at the centre of two Gerard-Hartline capillaries, so that the respiratory rate of one hemisphere could be compared directly with that of the other. With *Rana sylvatica* embryos a difference of 47 % was found in favour of the dorsal lip hemisphere. The fact that the difference to be expected was thus only 1.47 times was the reason why we were careful in nearly all our experiments to use dorsal lip and ventral ectoderm from the same gastrula.\*

It will be clear, therefore, that the present work on *Discoglossus* and *Amblystoma* is in agreement with the results of Waddington *et al.*, and not with those of Brachet and Shapiro. We believe, however, that the figures of the latter authors, though in appearance providing a sound proof of a differential oxygen consumption in favour of the dorsal lip region, do not really do so.

Although it is true that the omission of the four highest points for both regions in their series would make the difference between the two regions

\* We do not attach much significance to the difference of 20 % in favour of the dorsal lip found by Fischer and Hartwig for technical reasons referred to in the first paper of this series.

less statistically significant than it is ( $\frac{\text{diff.}}{\text{P.E. diff.}} = 9.96$ ), we nevertheless do not question the existence of a difference between the oxygen consumption of the two hemispheres. The Brachet-Shapiro apparatus deserves the greatest credit as the only one so far suggested whereby the oxygen consumption of regions of the amphibian embryo can be measured while it still remains intact. But when it is remembered that the gastrula is a relatively complex morphological structure rather than a homogeneous sphere, the interpretation of the difference observed by Brachet and Shapiro becomes more involved. When we were making our determinations of *Amblystoma* isolates we noticed a constantly recurring phenomenon; namely that in the manometers the oxygen uptake of the dorsal lip pieces would greatly exceed that of the ventral ectoderms, but that this difference vanished as soon as the figures for total N were obtained. Although the areas of the dorsal lip and ventral ectoderm isolates were approximately the same, the figures for total N revealed that, in general, the dorsal lip piece contained twice as much material as the ventral ectoderm. We attributed this fact to the circumstance that the dorsal lip region consists of a double layer (the tissue which has already invaginated, and that which is about to do so), while the ventral ectoderm has only one layer and is often thinner than either the archenteron roof or the as yet un-invaginated presumptive mesoderm.

If then we consider a gastrula as oriented in the Brachet-Shapiro apparatus we see that on the left of the vertical meridian there is ventral ectoderm, blastocoele cavity, and a certain amount of yolk endoderm, while on the right there are two layers of tissue above the archenteron, a certain amount of ectoderm at the ventral blastopore lip, and the major part of the yolk. The major part of the yolk endoderm must be situated in this hemisphere because the gastrula always floats blastopore downwards. If we disregard the yolk endoderm because of its low respiratory rate, it is clear that on the right-hand side there is considerably more than one hemisphere of highly respiring tissue on account of the invaginated material. Brachet and Shapiro were careful to try some experiments in which the gastrula was oriented so that the blastopore came equally in both hemispheres; they then found the oxygen uptakes to be exactly equal. In short, we accept the difference shown by them to exist between the two hemispheres of the gastrula, but we attribute it to the unequal amounts of actively respiring tissue in the two hemispheres, and not to a difference of respiratory rate between the dorsal lip region and the ventral ectoderm.

These interpretations, however, do not dispose of the differences in  $\text{CO}_2$  production found by Brachet (1936) between isolates from dorsal lip and ventral ectoderm of known total N content. An identical  $\text{O}_2$  consumption with a differing  $\text{CO}_2$  production can only mean a different respiratory quotient. This will be the subject of our next communication.

It is interesting to compare the absolute levels of  $Q_{\text{O}_2}$  here reported for the amphibian embryo with those for the mammalian egg. For the ripe unfertilized egg of the cow, Dragoiu, Benetato and Opreanu (1937) have found values ranging from 21 to 35. The contrast with those of the order of 0.5 or under for the amphibian embryo remains great even when we allow for the  $17^\circ$  difference in temperature, and must doubtless be due to the fact that the amphibian egg is highly lecithic, and that much yolk is present even in cells deriving from the animal pole. Immature amphibian eggs, which would contain less yolk, show a  $Q_{\text{O}_2}$  of  $-1$  to  $-2$ , according to Mestscherskaya (1935).

The same consideration applies to comparisons between the respiratory rate of gastrula regions and adult amphibian tissues. Thus tadpole epidermis, according to Erdmann and Schmerl (1926) and Börnstein and Klee (1927), has  $Q_{\text{O}_2}$  of from  $-1.21$  to  $-2.18$  (cf. the  $Q_{\text{O}_2}^N$  figures reported by Boell, Needham and Rogers for tadpole epidermis of  $-1$  to  $-2$ ). This contrasts with the  $-0.2$  to  $-0.3$  for ventral ectoderm (presumptive ventral epidermis) contained in the present paper. Börnstein and Klee found that skin from old frogs had a lowered respiratory rate (though not to the gastrula level) and that explants of skin had a much higher  $Q_{\text{O}_2}$ , up to  $-6.75$ . Comparable data for adult nervous tissue will be found in Winterstein and Hirschberg (1927) and Gerard (1927). The respiratory rate of the amphibian embryo is thus of about the same order as that of many adult invertebrate tissues; thus anemone tentacles were found by Kramer (1937) to have a  $Q_{\text{O}_2}$  of about  $-1$ .

The thanks of the authors are due to Professor Courrier for help in the obtaining of North African toads, and to the Government Grant Committee of the Royal Society which partially defrayed the cost of these researches. One of us (E. J. B.) held a Rockefeller Fellowship during the work.

#### SUMMARY

1. The Cartesian diver manometer has been adapted for the measurement of oxygen consumption.
2. The respiratory rate of the dorsal lip region and the ventral ectoderm from gastrulae of *Discoglossus* and *Amblystoma* appears to be identical.



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Morphogenesis and metabolism: studies with  
the Cartesian diver ultramicromanometer

IV. Respiratory quotient of the regions of  
the amphibian gastrula

By E. J. BOELL, HENRI KOCH AND JOSEPH NEEDHAM

*The Biochemical Laboratory, Cambridge*

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INTRODUCTION

No method has so far been published for obtaining the respiratory quotient of small pieces of tissue or living organisms the total gas turnover of which in the available experimental period is likely to be of the order of  $\frac{1}{16}$  cu.mm. In the present paper we shall describe such a method and give details of the results obtained with it on single isolated pieces of the regions of the amphibian gastrula.

Dixon (1934) and others have given sufficient reasons for rejecting all methods which do not determine the oxygen consumption and the production of carbon dioxide on the same piece of tissue, so that the simplest method on the usual large scale is that of Dickens and Šimer (1930) in which annular cups are used. As this necessitates the use of phosphate buffer solutions, which are regarded as unphysiological for work with mammalian tissues, the later more complicated methods in which bicarbonate is employed came into general use. For the present problem, however, in which the amphibian embryonic tissues studied remain perfectly normal in Holtfreter solution or even tap or river water, the difficulties caused by bicarbonate were avoided. At the same time, we believe that it should be by no means impossible to adapt the diver manometer to measurement of respiratory quotient even in bicarbonate medium.

## TECHNIQUE

(1) *Theory of the method*

In the last paper of this series it was shown that a drop of alkali could be made to remain just above the junction of the diver neck with the diver bulb if the neck was waxed. In this way, by absorption of  $\text{CO}_2$ , oxygen uptake could be measured. For the measurement of respiratory quotient all that it is necessary to do is to take a diver with a neck a little more than twice the normal length, and to place in it, when waxed, a drop of acid above the alkali drop and below the oil seal. During the first part of the experiment, the  $\text{CO}_2$  is absorbed by the alkali and a negative pressure results causing a progressive decrease in the buoyancy of the diver (measurable on the manometer). At the desired moment the diver in its vessel is subjected to a pressure of about 12 in. of mercury, so that both the alkali drop and the acid drop above it are forced down into the bulb, mixing with each other and killing the tissue. On removal of the special pressure the oil drop returns to its original position, and a positive pressure develops causing a rather quick increase in the diver's buoyancy. This is measured by the water manometer in the usual way, and when the buoyancy undergoes no further change, the amount of  $\text{CO}_2$  liberated is known and the respiratory quotient can be calculated. As in the Dickens-Šimer method, it is necessary to run a separate diver with a piece of tissue as similar as possible to the first, blowing down the drops at the beginning of the experiment, in order to ascertain approximately the amount of bound  $\text{CO}_2$ .

(2) *Dimensions and arrangement of the diver*

Respiratory quotients of divers, as used by us, have a volume of about  $40\lambda$  instead of about  $25\lambda$  for anaerobic glycolysis and oxygen consumption.  $V_F$  is  $0.4\lambda$  and the resulting  $V_G$   $25.6\lambda$ . The length of the neck is about 16 mm. instead of the 6 or 7 mm. as previously described. The neck is waxed.

The arrangement is shown in fig. 1. Immediately above the bulb containing tissue and Holtfreter solution without bicarbonate there is the drop of alkali,  $1.4\lambda$  of baryta. In the Dickens-Šimer method,  $m/6$  baryta is used, with  $2.5\text{ N HCl}$  in the side bulb, but when solutions with a six times difference of osmotic pressure such as these are placed in as near proximity as is demanded by microtechnique, distillation quickly occurs, and the drop of weaker solution may almost disappear. We therefore brought saturated baryta (about  $0.16\text{ M}$ ) to a more nearly equal osmotic

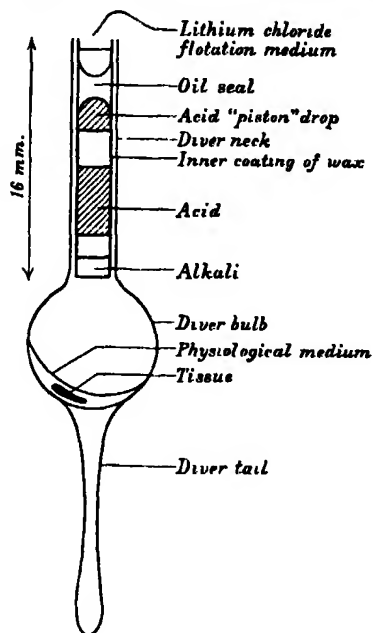


FIG. 1

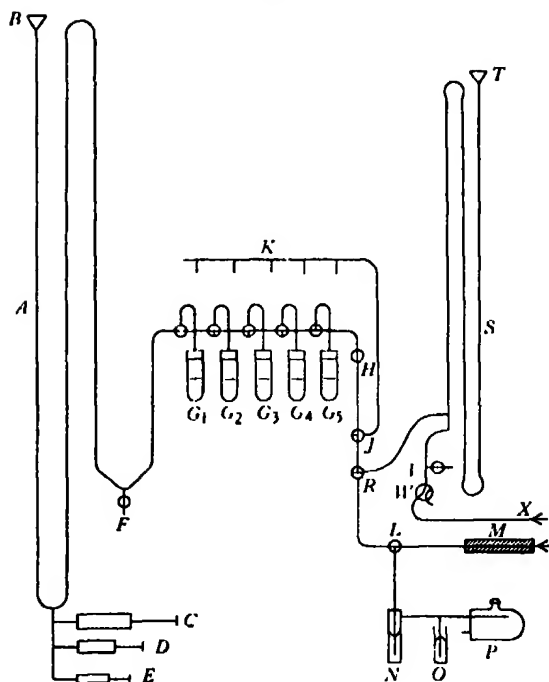


FIG. 2

pressure by the addition of sufficient sodium chloride to make it 2.4 N (0.285 g. NaCl to 20 c.c. saturated baryta). At the same time, to ensure an excess of acid to drive off the  $\text{CO}_2$ , we use 3.7  $\lambda$  of 2.5 N HCl adjacent but higher up the diver neck (see fig. 1). After another small interval there is placed a further 1.3  $\lambda$  of the acid immediately in contact with the oil seal. This we term the "piston" drop; its purpose will be referred to below. Above the oil seal there is the usual air bubble between it and the flotation medium of lithium chloride solution.

The amounts of the various drops are measured by their length in millimeters since after a normal coating of the neck with high melting-point paraffin wax, 1 mm. corresponds to 1.23  $\lambda$ .

### (3) Accessory pressure apparatus

The means used for subjecting the diver vessels to an additional pressure for the blowing down of the drops is shown in fig. 2, which should be compared with fig. 7 of the first paper of the series (Boell, Needham and Rogers 1938). The disposition of the water manometer, syringes for controlling it, diver vessels, and taps, is exactly the same as is there indicated, but at *R* is inserted a three-way tap. Whenever it is desired to blow down drops in a diver, the tap *F* is opened so as to safeguard the water manometer, and whichever vessel required is placed in connexion with *R* by turning its appropriate three-way tap and having taps *H* and *J* open to connect with *R*. Compressed air is now allowed to enter through *X*; under our conditions, at its maximum outflow to the room through *V*, a flow pressure of 2 or 3 in. is seen in the mercury manometer *S*, but this is insufficient to move the drops very far. *V* can now be closed so that the outflow goes through *W*, a three-way tap so arranged that the pressure of the finger or thumb can close it. The pressure is now cautiously and steadily applied, being steadily released as soon as the top of the acid drop has disappeared into the bulb of the diver.

If the divers are simply allowed to rest on the floor of the vessels they will be found to careen over as soon as the pressure from the mercury manometer is applied, and if this happens the drops will not flow down cleanly into the diver bulb. We therefore placed small holders, glass disks with a small length of glass tubing attached to them with de Khotinsky cement, at the bottoms of the vessels. The tails of the divers enter these tubes when they sink after each reading, and when the strong pressure is later applied, the divers are held firmly upright (see fig. 3).



FIG. 3

(4) *Proof of the correct return of the oil drop*

In order to establish the correct return of the oil drop after subjection to the accessory pressure a good many experiments were made. With divers filled with air it was easy to see that no significant difference in buoyancy took place before and after the moving of the oil seal by the accessory pressure and its return. More important were the tests with known amounts of bicarbonate.

Of a sodium bicarbonate solution giving 164.5 cu.mm./c.c., amounts of 1.5λ were placed in the diver bulbs, and after equilibrium had been attained, acid drops were blown down. The results follow.

Exp.	$\lambda \times 10^{-3}$	%
vi. 22	222	90
	260	106
vi. 25	244	100
	223	91
vi. 23	219	90
	245	99
vi. 24	243	99
	230	94
	238	97
vi. 29	247	100
	252	107
vii. 2	248	101
	246	100
Theoretical recovery		246.5

These figures show that the recovery of known amounts of  $\text{CO}_2$  introduced into the divers is practically quantitative (98 %).

During the course of these experiments a phenomenon was met with for which we have no certain explanation but which we were able to avoid by a special precaution. In certain cases the divers, instead of remaining at constant buoyancy if they contained water, or giving a good recovery of gas if they contained bicarbonate, showed a buoyancy decrease amounting to as much as - 6 cm. on the water manometer. We thought that this might be due to some mixture of the oil with the wax on the neck being blown down with the acid and forming a monolayer over the liquid in the bulb. If then a trace of strong acid remained in the neck, the water vapour in the bulb might be absorbed, and not replaced from below. In order to stop it, we adopted the plan of placing a "piston" drop of acid immediately under the oil seal. Whether the explanation was correct or not, the practice proved quite effective, and no instance of the phenomenon was seen in later experiments. The piston drop returns, of course, with the oil seal, after the accessory pressure is removed.

*(5) Introduction of tissue and rewaxing*

The diver is first filled with Holtfreter solution (without bicarbonate) in the usual way, and then, when submerged beside the gastrula isolates, the tissue was introduced into it. The excess Holtfreter solution is then removed.

It is absolutely essential, however, to rewax the diver neck after the introduction of the tissue. If this is not done, minute traces of mucus adhering to the wax will so seriously upset the menisci of the drops subsequently put in that they will prematurely flow together, or in the end the blowing down will not take place properly. Rewaxing is done very simply by holding the diver in such a position that a small length of iron wire heated slightly penetrates into the neck nearly as far as the junction with the bulb. The wire is conveniently held at an appropriate angle in a small stand and at the other end coiled over a microflame. In special tests it was found that if vaseline was placed inside the bulb it showed no tendency to melt, so there can be little danger of injury to the tissue unless the hot wire is allowed to penetrate beyond the neck.

It is also absolutely essential that the paraffin wax coating of the neck cease at the junction with the bulb. If it extends even a little way into the bulb, the drops will fail to come in contact with the clean glass surface when they are blown down, and it will be quite impossible to get them into the bulb.

*(6) Introduction of acid and alkali*

Since it is necessary that the amount of  $\text{CO}_2$  introduced into the diver in the form of carbonate in the baryta should be reduced to a minimum, in order to keep the blank value as low as possible, special precautions have to be taken in the filling of the divers with the solutions. It was found that the use of a stream of  $\text{CO}_2$ -free air under which the divers were filled, as in the technique of Linderström-Lang and Glick (1938), was quite insufficient, nor was the result much improved when the operation was done with a large glass screen intervening between the diver and the operator. Eventually the following technique was adopted as indispensable.

Baryta is drawn off from the lower layers of a saturated solution (to which salt has been added as described above) by an Agla micrometer syringe, in which it is permanently kept. The Agla syringe (Burroughs Welcome) holds just over 0.5 c.c. and is provided with a micrometer screw head with a Vernier scale. Graduations on the stem correspond to  $10\lambda$  and on the screw head to  $0.2\lambda$ . The Agla syringe, when filled with extreme care to exclude air bubbles, is mounted in a "wind-tunnel" of large-bore

glass tubing as shown in fig. 4*a*. The wind-tunnel, *A*, is so arranged that two tubes bring in at one end a strong stream of air rendered  $\text{CO}_2$ -free by passing through a soda-lime tower and bubbling through soda. These are shown at  $B_1$ ,  $B_2$ . The same stream runs also through a narrow opening,  $B_3$  (in actual practice taps have to be inserted in the companion streams in order to get a satisfactory flow through  $B_3$ ). The Agla syringe, *D*, is mounted together with the other tubes in the rubber stopper, *C*, which carries in addition a narrow-mouthed pipette to hold the acid (*E*). The Agla syringe is furnished with a capillary tip attached to it with wax. *G* is a small vessel containing mercury which closes the baryta tip when the apparatus is not in use; mercury is sucked a little way into the pipette and this effectually excludes any air.

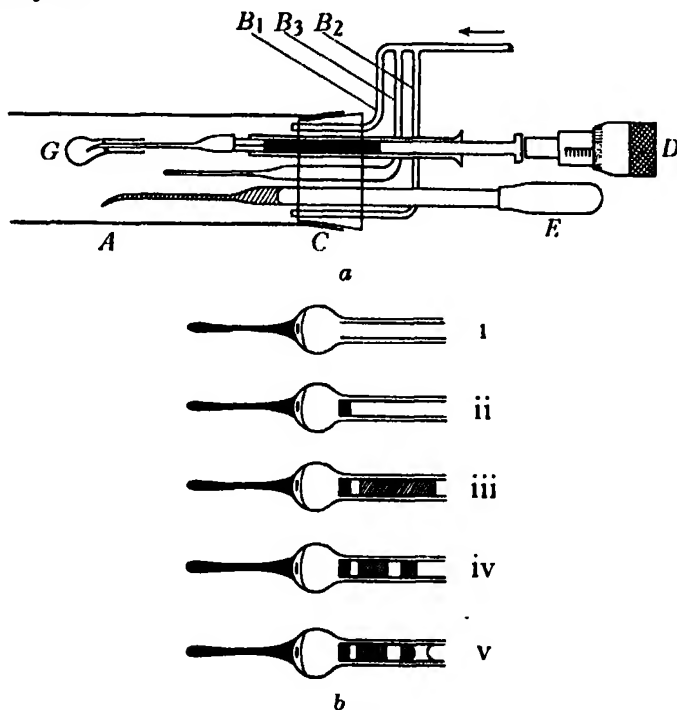


FIG. 4

The whole apparatus shown in fig. 4*a* is set up inside an old balance-case from which the ends have been removed and replaced with rubber diaphragms. These have holes cut to such a size that when the hands are inserted they fit well around the wrists. Through the balance-case there circulates an additional stream of  $\text{CO}_2$ -free air, so that if by any chance an eddy finds its way into the wind-tunnel no great harm will be done.



The filling of the divers takes place as follows. They are placed in order in a carrier inside the balance-case, and the streams of  $\text{CO}_2$ -free air turned on. After 5 min. the mercury seal is removed from the end of the baryta syringe reservoir and laid on the floor of the wind-tunnel. A small drop of baryta is expelled from the tip of the syringe, which is dried on a piece of clean filter-paper. Each diver in turn, which to start with is as shown in fig. 4*b* (i), is then held in the stream of  $\text{CO}_2$ -free air from the capillary jet,  $B_3$ , for half a minute, after which the requisite amount of baryta is injected into its neck from the baryta syringe. It now looks as in fig. 4*b* (ii). It is then held against the opening of the acid pipette and the remainder of its neck filled with acid, as in fig. 4*b* (iii). When all have been treated in this way it is safe to remove them from the balance-case, for the acid will protect the baryta from the  $\text{CO}_2$  of the atmosphere. Naturally the baryta syringe tip must be carefully protected by the mercury vessel,  $G$ , before this is done. The excess acid in the neck is now removed at ease in the air, the piston drop is placed in position, as in fig. 4*b* (iv), and after the oil seal has been added, the diver is ready to be placed in the flotation medium (fig. 4*b* (v)).

With these conditions the solutions blank can be made so small as to be beneath the range of measurement even with the diver manometer, that is to say, under one thousandth of 1 cu.mm.

#### (7) *Reading of divers*

Divers of the volume and neck length required in the present experiments were found to behave in the vessels somewhat more sluggishly than smaller divers. It is therefore advantageous to substitute for the usual magnifying glass outside the bath a travelling microscope of long focus arranged on so stable a runway that its cross wires may be used for sighting the divers instead of the usual lines etched on the glass of the vessels. By this means minute upward or downward tendencies of the diver near its equilibrium position may be seen, and therefore corrected, more quickly than is otherwise possible. This will enhance the accuracy of the readings. All the data in the present paper, however, were obtained without the aid of a travelling microscope.

#### (8) *Cleaning of divers*

At the conclusion of the experiment the divers are handled as described in the first paper of this series, except that hot toluene is first run through them. This removes the wax coating of the neck in a fraction of a second.

(9) *Determination of constant*

Since the oil drop is in these experiments much farther away from the gas space in which the changes are occurring than in the  $Q_L^N$  or  $Q_{O_2}$  divers, and since it is separated from the gas space by a relatively long column of liquid, it is not included in  $V_F$ . The alkali, the acid, and the piston acid are, however, included. The distance between the lithium chloride meniscus and the lower oil meniscus is measured in every case in millimeters, and the volume in  $\lambda$  is called the dead space ( $ds$ ). A sample calculation of constant is here given (cf. the formula in Boell, Needham and Rogers 1939, p. 332).

	$\lambda$	
$V$ (after waxing)	42.13	
$ds$	4.92	
$V - ds = V'$	37.21	
$V_F$	9.8	
$V_G$	27.41	
$V_G \times t/T$	—	25.6
$V_F \times \alpha_{O_2}$	—	0.3
$K_{O_2}$	—	16.5
$V_F \times \alpha_{CO_2}$	—	8.6
$k_{CO_2}$	—	21.8

(10) *Tests of the method*

Two tests of the method were made on living material of which the behaviour was so well known as to constitute a check on the whole procedure. The first was yeast in glucose to give a respiratory quotient of unity; the second was an embryo of the grasshopper, *Melanoplus differentialis*, which is known (Boell 1935) to give invariably a quotient of 0.7 or a little below.

For yeast two sample protocols follow.

## Exp. vii 4

1.87  $\mu$ g. dry weight yeast,  $t$  2 hr. 20 min.

$h_{O_2}$ (cm.)	— 13.7	— 14.2
$h_{CO_2}$ (cm.)	+ 15.3	+ 17.1
$h_{\text{bound } CO_2}$ (cm.)	+ 4.9	+ 4.9
$h_{\text{resp. } CO_2}$ (cm.)	+ 10.4	+ 12.2
$k_{CO_2}$	20.6	23.7
$k_{O_2}$	16.1	19.2
$x_{CO_2} (\lambda \times 10^{-3})$	+ 214	+ 289
$x_{O_2} (\lambda \times 10^{-3})$	— 220	— 272
R.Q.	0.97 <sub>6</sub>	1.06

In this case the gas turnover was between  $\frac{2}{10}$  and  $\frac{3}{10}$  cu.mm. *in toto*.

The next two protocols are for grasshopper embryos. They were removed from their shells by cutting off the anterior tip of the egg and allowed to remain in Belar solution for about an hour before being introduced, one each, into the divers.

## Exp. vii 10

1 grasshopper embryo at end of diapause,  $t$  2 hr. 20 min.

$h_{0.5}$ (cm.)	- 25.0	- 23.1
$h_{1.0}$ (cm.)	+ 18.4	+ 16.5
$h_{\text{bound } 0.5}$ (cm.)	+ 5.0	+ 5.0
$h_{\text{respir. } 0.5}$ (cm.)	+ 13.4	+ 11.5
$k_{0.5}$	18.95	19.6
$k_{1.0}$	14.6	15.0
$x_{0.5}$ ( $\lambda \times 10^{-3}$ )	+ 254	+ 226
$x_{1.0}$ ( $\lambda \times 10^{-3}$ )	- 365	- 347
R.Q.	0.70	0.65 <sub>2</sub>

Respiratory quotients for such embryos obtained by the usual methods always vary between 0.7 and 0.6.

## RESULTS

The results are summarized in Table I and fig. 5. In fig. 5 the points are arranged according to morphological age according to Harrison numbers. Before beginning the experiments it had been expected that the blastula pieces would give respiratory quotients in the neighbourhood of 0.7 (from the work of Brachet 1934 and of Białaszewicz and Błędowski 1915, on the intact embryos) and that neurala isolates would give quotients of unity (again from the work of Brachet 1934). Both these expectations were fulfilled. But when the distribution of the points between these stages, i.e. during the gastrulation process, were plotted, it was seen that the dorsal lip region has a much more marked tendency to unity than the ventral ectoderm. It is not indeed certain that the ventral ectoderm ever reaches unity before it becomes completely underlain by mesoderm radiating from the sides of the blastopore opening. Pure ventral ectoderm cannot be obtained after the  $\frac{3}{4}$ -moon yolk-plug stage, so in the diagram the subsequent area is shaded.

To show that oxygen diffusion is not a limiting factor in these experiments, we plotted the respiratory quotient against the oxygen consumption, obtaining a random distribution. There was no sign that lower oxygen uptakes tended to give higher respiratory quotients.

TABLE I. RESPIRATORY QUOTIENTS OF REGIONS OF *AMELYSTOMA* GASTRULAE

Exp. no.	Stage	Harrison no.	Region	t hr.	Total N μg.	$-x_{1/2}$ $\lambda \times 10^{-3}$	$+x_{\text{exp. min}}$ $\lambda \times 10^{-3}$	R.Q.	$Q'_{1/2}$
ARQ 2 2	fm yp	10½	dl	3	22.0	-244	+262	1.07	3.7
3	fm yp	11	dl	3	—	-225	+215	0.96	—
4	fm yp	11½	dl	3	26.8	-272	+223	0.82	3.4
ARQ 3 2	fm yp	10½	ve	3.2	—	-287	+230	0.84	—
3	fm yp	10½	ve	3.2	17.25	-194	+214	1.15	3.8
4	fm yp	10½	ve	3.2	16.15	-202	+158	0.79	4.2
ARQ 4 2	fm yp	10½	ve	3.9	20.8	-335	+273	0.81	4.0
3	½-fm yp	10½	ve	3.9	18.25	-213	+195	0.92	2.9
4	fm yp	11	ve	3.9	6.1	-64	+53	0.83	2.6
ARQ 5 2	fm yp	10½	dl	3.2	—	-236	+239	1.01	—
3	fm yp	10½	dl	3.2	—	-238	+241	1.01	—
4	fm yp	11½	dl	3.2	—	-318	+302	0.95	—
ARQ 6 1	fm yp	10½	dl	3	28.9	-297	—	—	3.4
2	fm yp	10½	ve	3	13.3	-197	—	—	4.8
3	fm yp	10½	dl	3	12.1	-392	+406	1.03	10.8
4	fm yp	10½	ve	3	8.35	-179	+82	0.79	7.2
ARQ 7 3	Blastula	9	brf	3.5	14.6	-210	+148	0.71	4.1
4	Blastula	9	brf	3.5	8.3	-125	+100	0.80	4.3
ARQ 8 2	Neurula	18	cnf	3	16.9	-250	+258	1.03	4.9
3	Neurula	18	cnf	3	—	-358	+381	1.06	—
4	Neurula	18	cnf	3	17.5	-240	+256	1.06	4.6

Abbreviations: fm yp, quarter-moon yolk-plug gastrula; ½m yp, half-moon yolk-plug; ¾m yp, three-quarter-moon yolk-plug; fm yp, full-moon yolk-plug; dl, dorsal lip region; ve, ventral ectoderm; brf, blastocoel roof; cnf, closing neural folds. Brackets indicate that both pieces came from the same gastrula.

A sample protocol follows:

Exp. vii 7

Ventral ectoderm isolates of *Amblystoma* gastrulae,  $t$  4 hr.

Harrison no. ...	10 $\frac{1}{2}$	10 $\frac{3}{4}$	11
$h_{0\frac{1}{2}}$ (cm.)	- 23.5	- 13.3	- 5.4
$h_{1\frac{1}{2}}$ (cm.)	+ 18.6	+ 13.1	+ 3.8
$h_{\text{bound } 0\frac{1}{2}}$ (cm.)	+ 4.6	+ 4.2	+ 1.4
$h_{\text{rev. } 0\frac{1}{2}}$ (cm.)	+ 14.0	+ 8.9	+ 2.4
$k_{1\frac{1}{2}}$	19.5	21.95	22.1
$k_{0\frac{1}{2}}$	14.25	16.0	16.9
$x_{1\frac{1}{2}} (\lambda \times 10^{-3})$	+ 273	+ 195	+ 53
$x_{0\frac{1}{2}} (\lambda \times 10^{-3})$	- 335	- 213	- 64
R.Q.	0.81 $\frac{1}{2}$	0.92	0.83

It will be seen that one of these was obtained on a gas turnover of only one twentieth of 1 cu.mm.

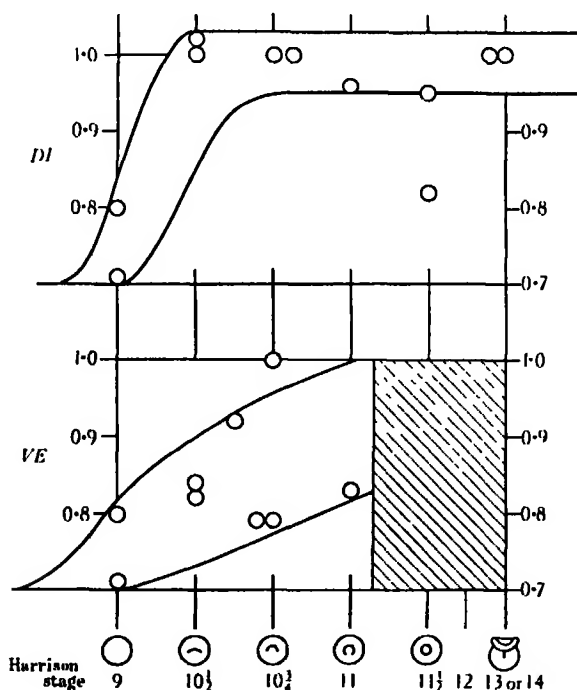


FIG. 5. Respiratory quotients of isolated pieces of *Amblystoma* gastrulae plotted against morphological age according to the progress of gastrulation. N.B. Quotients above unity are scaled down to unity for diagrammatic purposes (see Table I). The shaded area, in the case of ventral ectoderm, indicates that in the late stages of gastrulation pure ventral ectoderm can no longer be obtained.

It is interesting to compare the rates of oxygen consumption noted in the present series of experiments with those previously reported for gastrula isolates (Boell and Needham 1939). The averages work out as follows:

	$Q'_{O_2}$
Blastula, roof of blastocoele	4.2
Gastrula, dorsal lip region	5.3
Gastrula, ventral ectoderm	4.2
Neurula, closing neural folds	4.8

We are not inclined to regard these differences as significant. If the two highest values for dorsal lip and ventral ectoderm be omitted (both came from one gastrula in Exp. ARQ 6), the averages become 3.5 and 3.2 respectively, i.e. very close to those reported in the preceding paper of this series.

The difference between dorsal lip region and ventral ectoderm as regards anaerobic glycolytic rate and the identity of their metabolic level as regards oxygen consumption may have appeared contradictory, or at least paradoxical. But in the present experiments we see that during invagination the respiratory quotient of the regions changes. We have to do, therefore, with a progressive alteration of the *quality* of metabolism during gastrulation, and not with its *quantity*.

One of us (H. K.) was on leave from the University of Louvain, and another (E. J. B.) held a Fellowship of the Rockefeller Foundation during the course of this work. The expenses of the work were partially defrayed by a grant from the Government Grant Committee of the Royal Society.

#### SUMMARY

1. The Cartesian diver manometer has been adapted for the measurement of respiratory quotient.

2. During amphibian gastrulation (experiments on *Amblystoma*) the dorsal lip region shows a greater trend towards a respiratory quotient of unity than does the ventral ectoderm. Though the respiratory quotient of the latter rises, it is doubtful whether it attains unity before it is completely underlain by mesoderm.

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## The use of chemical potentials as indices of toxicity

BY J. FERGUSON, *Castner-Kellner Alkali Company, Runcorn**(Communicated by A. J. Clark, F.R.S.—Received 1 December 1938)*

## INTRODUCTION

A number of investigations have been published in which attempts are made to correlate the chemical or physical properties of substances with the intensity of their toxic action. Among these there may be instanced the work of Moore (1917) on the toxicity of fumigants towards insects, the extensive work of Tattersfield and his collaborators on contact insecticides and fumigants (Tattersfield and others 1920, 1925, 1926, 1927), the bacteriological investigations of Tilley and Schaffer (1926, 1928) and of others (Coulthard, Marshall and Pyman 1930; Dohme, Cox and Millar 1926; Klarman, Gatyas and Shternov 1931), work on fungicides (Morris 1926) and the studies of Stiles and his co-workers on plant tissue (Stiles and Stirk 1932; Stiles and Rees 1935).

In each of the investigations just quoted a more or less wide range of chemical compounds was examined. The experiments consisted in the determination of the molar concentrations of the compounds which produced equi-toxic effects on a selected organism. The toxicities of the compounds studied were then usually assumed to be inversely proportional to the values of these equi-toxic concentrations.

The equi-toxic concentrations were measured either in an aqueous solution in which the organism was immersed or, in the case of fumigants, in the gaseous atmosphere surrounding the organism. The toxic concentration, therefore, in no instance was measured in the centres directly affected by the toxic agent.

It can readily be shown that in very many cases of physiological action, an equilibrium must exist between the concentration of the active substance in the circumambient medium or in the body fluids of the organism and its concentration in the particular cells or structures directly affected by it. It follows, therefore, that in those cases the measured equi-toxic concentrations must be markedly influenced by the phase distribution relationships of the various toxic substances. In other words, the measured toxic concentration, though usually regarded as an index of toxicity, is in reality a function of the intrinsic toxicity of the substance and of its distribution equilibrium. Only when the effect of phase distribution is allowed for can intrinsic toxicities be compared, and can valid deductions be drawn regarding possible relationships between chemical constitution and physiological action. Were such information available, the elucidation of the mechanism of action would be greatly facilitated.

The great influence of phase distribution relationships in determining the values of physiologically active concentrations is of course recognized in the Meyer-Overton lipoid theory of narcosis. In the later form of this theory adopted by K. H. Meyer (Meyer and Hemmi 1935), it is assumed that isonarcotic effects are produced by the most diverse substances when their molar concentrations in the cell lipoids are identical. The widely different isonarcotic concentrations measured in the external phase on this theory, therefore, are determined entirely by the value of the lipoid/external phase distribution coefficient. It cannot, however, be said that the data produced by Meyer in support of his view enable such a conclusion to be drawn. The theory has also been adversely criticized on experimental grounds (Winterstein 1926).

The purpose of the following paper is to present a method of comparing the toxicities of different substances, free from any hypothesis, which, when applicable, eliminates entirely the disturbing effect of phase distribution on the comparison. It will be shown that the toxic indices so determined for any one organism lie within a relatively narrow range of values, though the measured toxic concentrations may vary very widely from substance to substance.

The method adopted is to use, instead of the measured concentrations, the chemical potentials of the substances in a phase in equilibrium with



that phase, (whatever it may be), which is the seat of toxic action. If a true equilibrium exists, the chemical potential of the toxic substance must be the same in all phases partaking in the equilibrium. Hence the chemical potential of the toxic substance at the actual-point of attack is known.

### EQUILIBRIUM RELATIONS

In narcotic action and in certain drug actions the maximum physiological effect is very rapidly attained and remains at the same level of intensity for considerable periods while the organism is in contact with a constant concentration of the drug or narcotic. On removal at any time to drug-free surroundings recovery is rapid. It is to be concluded that in these cases of reversible action an equilibrium must exist between the concentration of the substance in the external phase and its concentration in the affected phase within the organism.

The most striking evidence that such physical equilibria exist and determine the value of the physiologically active concentrations not only in cases of reversible physiological action such as narcosis, but in non-reversible actions such as the killing of bacteria and insects, is supplied by the numerous relationships between physical properties and toxic action detected in some homologous series. This has been pointed out by Meyer and Hemmi (1935) for narcotic action.

It is well known that for any one organism the equi-toxic and equi-narcotic concentrations of the members of some—but not all—homologous series decrease as the series is ascended—a generalization known as Richardson's rule. The decrease in toxic concentration, as was first shown by Fühner (1904), proceeds in a definite quantitative manner. The equi-toxic concentrations of the successive members are proportional to the reciprocals of the geometric progression 1, 3,  $3^2$ ,  $3^3$ , .... The value of the multiplying factor varies to only a small extent according to the particular series tested. In straight chain series it seldom differs much from 3. Examples of the phenomenon are the narcotic action of the alcohols, alkyl acetates and ketones on tadpoles (Overton 1901), the disinfectant action of the primary alcohols, ketones, amines and para-phenols towards *Bacillus typhosus* (Tilley and Schaffer 1926, 1927, 1928), the haemolysis of ox blood by urethanes, alcohols, esters and ketones (Fühner and Neubauer 1907), the contact insecticidal action of the alcohols (O'Kane and others 1930), the narcotic action of paraffin hydrocarbons on mice (Fühner 1921), the toxicity of alcohols and esters to potato tuber (Stiles and Stirk 1932).

As a consequence of this logarithmic rate of decrease, the measured toxic concentrations of the higher homologues of a series are relatively extremely low. Thus, Clark (1933) found that the concentrations of methyl alcohol and dodecyl alcohol solutions producing the same effect on a frog's heart stood in the ratio of  $(3.11)^{11} = 266,000$ . The extremely rapid decrease in toxic concentration does not, however, continue indefinitely. Sooner or later a homologue of maximum toxicity is reached, and beyond it the higher homologues are either feebly toxic or non-toxic. Examples of this sharp cut-off of toxicity can be found in the data of Coulthard, Marshall and Pyman (1930), Clark (1933), Tattersfield and Roberts (1920), Bousquet, Salzberg and Dietz (1935), etc.

In homologous series most physical constants show an approximately equal change on passing from one member to the next. There are, however, certain properties which behave similarly to the toxic concentration. These include water solubility, distribution between immiscible phases, capillary activity and vapour pressure. Water solubility in homologous series decreases in a geometric progression as the number of methylene groups increases in arithmetical progression, a fact first noted by Fühner (1924). The ratio of the molecular water solubility of one member to the next approximates to 4-4.5. The relation involving capillary activity is, of course, Traube's rule.

To illustrate these relationships reference may be made to fig. 1 in which the logarithms of the water solubilities, the isocapillary concentrations, the vapour pressures and the concentrations toxic to *B. typhosus* of the normal primary alcohols are plotted against the number of carbon atoms. The data other than the toxicity data have been taken from Landolt-Börnstein, *Physikalische Tabellen*. They all refer to 25° C. The figures for the concentrations of the alcohols toxic to *B. typhosus* have been calculated from the molecular phenol coefficients determined by Tilley and Schaffor (1926).

It will be noted from fig. 1 that the logarithms of the concentrations, etc. lie on straight lines when plotted against the number of carbon atoms in the molecule, except in the case of methyl alcohol, and it is further apparent from the existence of this exception that a very close correlation exists between certain physical properties and the toxic concentration.

In fact the toxic concentration  $C_r$  can be calculated with great accuracy by expressions of the form

$$C_r = kS_r^{1/n} \quad \text{or} \quad C_r = kp_r^{1/n},$$

where  $S_r$  is the solubility in mol./litre of the  $r$ th member of the series,  $p_r$  the vapour pressure,  $k$  and  $1/n$  being constants,  $n$  being greater than unity.

The close correlation of toxic concentration and vapour pressure in homologous series does not appear to have been noted before.

K. H. Meyer and H. Hemmi (1935) in discussing theories of narcosis have pointed out that these close correlations in homologous series between narcotic concentration and the properties of water solubility, vapour pressure and surface activity, indicate that an equilibrium exists between the concentrations of the narcotics in the surroundings where they are

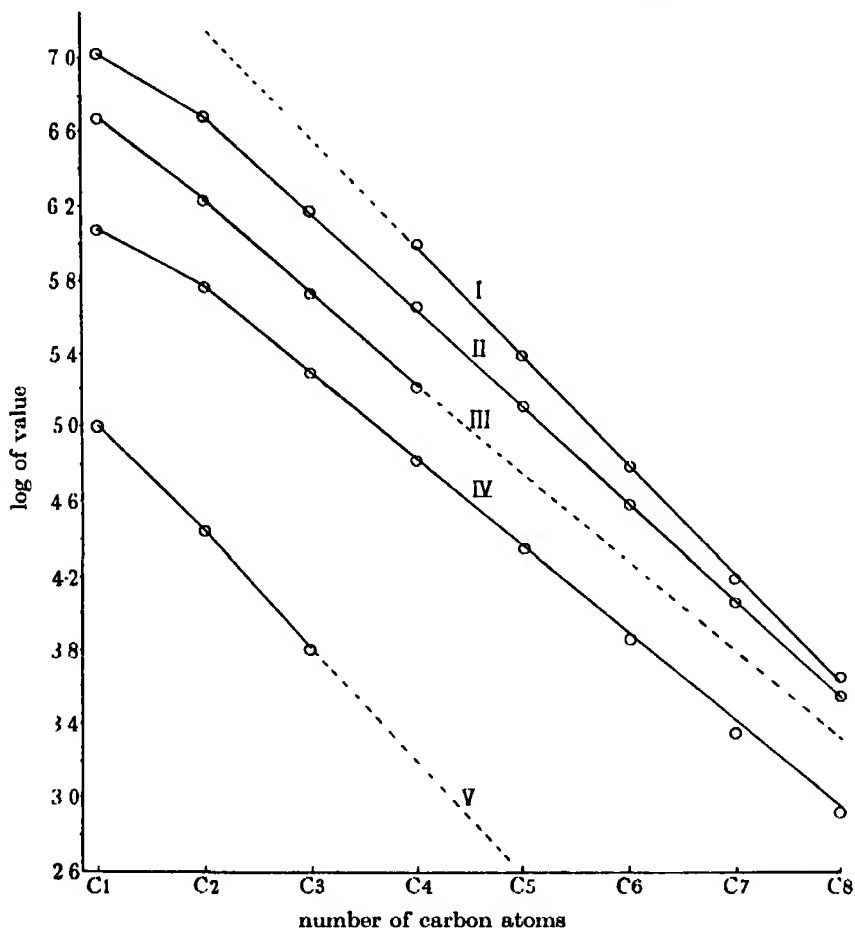


FIG. 1. Properties of normal primary alcohols.

- I. Solubility (mol.  $\times 10^{-6}$ /litre).
- II. Toxic concentration for *B. typhosus* (mol.  $\times 10^{-6}$ /litre).
- III. Concentrations reducing s.r. of water to 50 dynes/cm. (mol.  $\times 10^{-6}$ /litre).
- IV. Vapour pressure at 25° (mm.  $\times 10^4$ ).
- V. Partition coefficient between water and cotton seed oil ( $\times 10^3$ ).

measured and in the cell elements whose affection determines the narcosis.

For all these constants are expressions of a distribution between heterogeneous phases. Solubility is the expression of the distribution of a substance between the pure, or more correctly the relatively pure, solid or liquid phase, and its saturated solution; vapour pressure corresponds to the distribution between the pure solid or liquid phase and its vapour; surface activity is the expression of the distribution of a solute between the surface layer of a solution and its main bulk.

Since the molar toxic concentrations in a homologous series change on ascending the series not by equal steps but that instead their logarithms decrease by equal steps, it is to be concluded that they are largely determined by a distribution equilibrium between heterogeneous phases—the external circumambient phase where the concentration is measured and a biophase which is the primary seat of toxic action. As K. H. Meyer and H. Hemmi (1935) in dealing with narcosis have pointed out, this deduction is valid without having to specify the nature of the affected biophase. It may be lipoid or some other homogeneous phase, or it may be a surface layer, i.e. the equilibrium may be an adsorption equilibrium.

The reason for the logarithmic change of distribution coefficients in homologous series is enlightening. It was first stated by Langmuir as an explanation of Traube's rule (Langmuir 1917) and has been extended by Frumkin (1925). It can be readily shown (see Lewis and Randall, *Thermodynamics*) that when a substance is distributed in equilibrium between two immiscible phases the partition coefficient  $k$  is given by the following expression:

$$\log k = (\bar{F}_2^\circ - \bar{F}_1^\circ) \frac{1}{RT},$$

where  $\bar{F}_1^\circ$  and  $\bar{F}_2^\circ$  are the partial molal free energies of the substance in its standard states in phases I and II respectively.

In other words, the linear relation found in homologous series between the logarithms of various types of distribution coefficient and the number of carbon atoms in the molecule simply means that there is a constant increment for each successive homologue in partial molal free energy difference between the standard states in the two phases.

#### TYPES OF TOXIC ACTION

The existence of an equilibrium between the external and internal concentrations of substances exerting a physiological effect leads to two important consequences.

Firstly, when an equilibrium exists, and when the physiological effect is a function of the external concentration, it may be concluded that an irreversible chemical reaction with the constituents of the living cell does not occur. Nor can the results be explained by a heterogeneous chemical equilibrium of the diphenylamine picrate type (Walker and Appleyard 1896). On the contrary, it is justifiable to assume that the primary action is of a physical character. It may, for example, consist of adsorption of the substance on certain cell structures, or solution in the lipoids, or coagulation of cell proteins, etc., Narcosis has, of course, long been recognized to be due to a physical action of the narcotic substances (Hefter 1923). Narcotics act as whole molecules. The relations noted in homologous series indicate that in many other reactions between chemical substances and living matter, e.g. bactericidal and insecticidal action, the primary mechanism is of a physical character. The primary process is reversible and the irreversible effects culminating in death of the organism must be due to secondary processes.

The existence of direct correlations between solubility or vapour pressure and toxic concentration may therefore be used as a criterion to determine if the toxicity of a group of substances is to be referred to a physical or chemical mechanism.

All substances apparently can exert a physiological action by a physical mechanism. Even an inert substance like nitrogen at a sufficiently high pressure will induce narcosis (Meyer and Hopff 1923). The physical effect, however, may be, and frequently is, masked by a specific chemical effect. In some homologous series the lower members are to be regarded as chemically toxic. Chemical reactivity predominates. But, as the series is ascended, physical toxicity will come more and more into evidence until it swamps the chemical effect. The first members, therefore, will be approximately of the same apparent "toxicity" (as measured by the equi-toxic concentrations in the external phase), or will show a decreasing "toxicity" as chemical reactivity declines on ascending the series. At some point in the series physical toxicity will become predominant and the succeeding members will exhibit the rapid decrease in equi-toxic concentration normally found in homologous series.

An example of the appearance of the homologous effect is given by Dent's data (1932) on the toxicity of the aldehydes to the potato tuber (Table I).

von Oettingen (1936) found that the toxicity of the alkyl thiocyanates to mice decreased from methyl to hexyl thiocyanate. The decrease was shown to be correlated with the simultaneous decrease in the ease of splitting of hydrocyanic acid from the molecule by the organism.

TABLE I

Aldehyde	Relative toxicity per mol.
Formaldehyde	1.00
Acetaldehyde	0.64
Propionaldehyde	1.28
Butyraldehyde	1.54
Valeraldehyde	7.27

## CHEMICAL POTENTIAL AS A TOXIC INDEX

Granting that in cases of physical toxicity an equilibrium exists between the external and internal concentrations, an interesting deduction can be drawn. The chemical or thermodynamic potential of the toxic substance must be the same in each phase. Since the thermodynamical potential can be measured in the external phase, its value in the phase affected is known. The thermodynamical potential to be used here is the partial molal free energy of the substance referred to a standard state.

For perfect gases or perfect solution the partial molal free energy of a substance  $\bar{F}$  in any given state is given by the equation

$$\bar{F} = F_0 + RT \log C,$$

where  $F_0$  is the partial molal free energy in the standard state and  $C$  is the molal concentration of the substance.

With non-ideal solutions, etc., it is convenient to introduce in place of the concentration  $C$ , the function known as "activity" which is defined by the equation

$$\bar{F} = F_0 + RT \log a.$$

If the activity of the pure toxic substance is put equal to unity and its activities in the two phases referred to this standard state, then the activities of the toxic substance in the two phases are equal and its activity in the phase, whatever it may be, which is the seat of the toxic action is known by measurements of the activity in the external phase. It is suggested that activities rather than concentrations should be used as toxic indices for comparative purposes.

Few measurements exist of the activity of organic non-electrolytes in solution. The activities of the alcohols in aqueous solution at several concentrations have been determined by Butler, Thomson and MacLennan (1933). The activity-concentration relationship is also known for methyl acetate, acetone, pyridine, aniline, in aqueous solution (Landolt-Börnstein, *Tabellen*).

When such measurements are lacking it is possible sometimes to obtain a value for the activity to a greater or less degree of approximation. Thus if the toxic agent is applied to the test organism as a vapour, e.g. in insect fumigation experiments, the activity is given with fair accuracy by  $p_i/p_s$ , where  $p_i$  is the partial pressure of the fumigant, in the fumigation space calculated from the dosage in mol./litre and  $p_s$  the saturated vapour pressure of the substance at the temperature of the experiment. When the toxic agent is applied in solution, and is a substance of limited solubility, the activity at the toxic concentration can be very approximately put equal to  $S_i/S_0$ , where  $S_i$  is the molar concentration of the toxic solution and  $S_0$  its solubility in mol./litre (or mol. fractions). Though, of course, considerable errors may be introduced by this latter procedure, it will be sufficiently accurate for the use made of it in what follows.

Utilizing the measurements of Butler, Thomson and MacLennan (1933), the activities of the alcohols in equi-toxic solutions for various toxic and narcotic actions have been calculated from published results.

These are given in Table II.

TABLE II

	Inhibition of development of sea urchin eggs (Fuhner 1904)		Bactericidal concentration <i>B. typhosus</i> (Tilley and Schaffer 1926)		Fumigation rod spider (Read 1932)	
	Mol./l.	Activity	Mol./l.	Activity	Mol./l.	Activity
Alcohol						
Methyl	0.719	0.019	10.8	0.33	0.00017	0.26
Ethyl	0.408	0.026	4.86	0.32	0.00010	0.32
Propyl	0.136	0.034	1.50	0.34	0.000047	0.42
Butyl	0.0454	0.043	0.45	0.37	0.000017	0.47
Amyl	0.0204	0.070	0.13	0.52	0.0000081	0.56
Hexyl	—	—	0.039	0.63	—	—
Heptyl	0.00172	0.112	0.012	0.74	—	—
Octyl	0.00051	0.113	0.0034	0.88	—	—
	Tadpole narcosis (Overton 1901)		Haemolysis of ox blood (Fuhner and Neubauer 1907)			
	Mol./l.	Activity	Mol./l.	Activity		
Alcohol						
Methyl	0.57	0.014	7.34	0.22		
Ethyl	0.29	0.020	3.24	0.22		
Propyl	0.11	0.029	1.08	0.24		
Butyl	0.038	0.038	0.318	0.27		
Amyl	—	—	0.091	0.31		
Hexyl	—	—	—	—		
Heptyl	—	—	0.012	0.77		
Octyl	—	—	0.004	0.87		

If any one column is taken in Table II, there will be noted a very rapid decrease in the toxic concentration measured in the circumambient medium as the series is ascended. Thus the toxic concentration of octyl alcohol to *B. typhosus* is about 1/3000 that of methyl alcohol. The activity, however, while increasing, shows only a small change, hardly trebling in value in passing from methyl to octyl alcohol.

This relatively small change in the activity, increasing as the series is ascended, is generally observable in homologous series. Further examples are given in Tables III and IV.

TABLE III. ISONARCOTIC AND ISOHAEMOLYTIC CONCENTRATIONS AND ACTIVITIES OF ALKYL ACETATES

Substance	Tadpole narcosis (Overton 1901)		Haemolysis (Fühner and Neubauer 1907)	
	Mol./l.	Activity	Mol./l.	Activity
Methyl acetate	0.08	0.026	1.15	0.43
Ethyl acetate	0.03	0.035	0.47	0.55
Propyl acetate	0.01	0.045	0.16	0.71

TABLE IV. ISONARCOTIC CONCENTRATIONS AND ACTIVITIES OF PARAFFIN HYDROCARBONS FOR MICE (FÜHNER 1921)

Substance	Isonarcotic conc. mol./l.	$p_i/p_s$ (activity)
Pentane	0.0052	0.29
Hexane	0.0017	0.34
Heptane	0.00064	0.44
Octane	0.00032	0.82

It will be apparent, if a series is ascended sufficiently far, the rate of increase in toxic activity being maintained, that a member will be reached exerting the same toxic effect as the lower members at an activity near  $10^{-6}$  unity, i.e. only when it is present as a saturated vapour or as a saturated solution. Beyond this member the further members of the series will be non-toxic or only feebly toxic under the same experimental conditions.

This is in fact the "cut-off" in toxicity already referred to. The "increase of toxicity" of fumigants as the boiling point rises, followed by a sharp decline beyond a critical boiling point noted by Moore (1917) and Tattersfield and Roberts (1920), is a further example.

The position of the cut-off in any series will depend on the resistance of the organism to the toxic agent. Very resistant organisms will cause the cut-off to appear early in the series. This is made clear by examination of



fig. 2. In this, Tilley and Schaffer's data for the toxic concentrations of the primary alcohols to both *B. typhosus* and *Staphylococcus aureus* are plotted on a logarithmic scale against solubility.\*

Since *Staphylococcus aureus* is a more resistant organism than *B. typhosus*, the intersection of the toxic concentration line with the saturation line

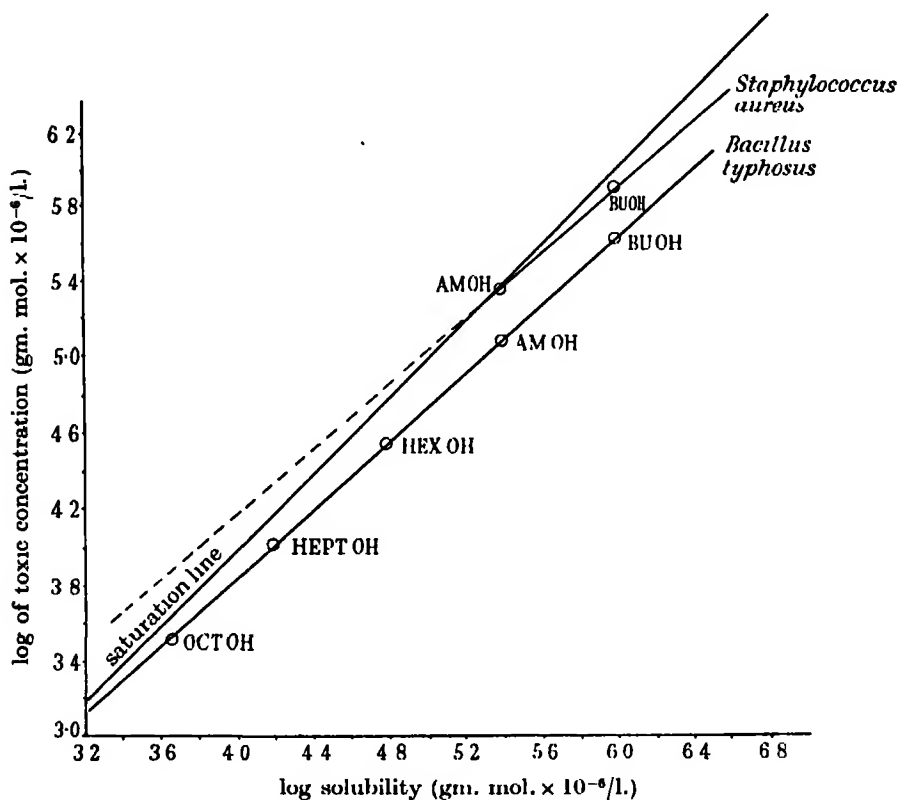


FIG. 2. Bactericidal concentration v. solubility for normal primary alcohols.

occurs earlier in the series. In agreement with this Tilley and Schaffer could obtain no phenol coefficients for alcohols higher than amyl alcohol when working with the former organism.

The above considerations satisfactorily explain the so-called "quasi-specific effect" of the bactericidal and fungicidal action of homologous

\* Since Tilley and Schaffer report only molecular phenol coefficients, it has been necessary to convert their results to molar concentrations to assume that in the technique and under the conditions used by them phenol killed *B. typhosus* at a dilution of 1 in 105. Any moderate error in this assumption does not affect the argument. The same assumption was made in constructing fig. 1.

series referred to by Klarman, Shternov and Gatyas (1934). They observed the usual increase of the toxicities of the alkyl derivatives of ortho and para chlorphenols with increasing molecular weight up to a maximum followed by a sharp decrease. The point of maximum toxicity varied according to the organism tested and from their results it is apparent that the maximum occurred earlier the greater the resistance of the organism. They attempt to divide organisms into two classes according as to whether they exhibit this "quasi-specific" effect or not and to correlate the difference with their response to gram stain. The simple explanation given above is all that is necessary.

Another example of the selective effect is furnished by the action of the alkyl resorcinols on differently resistant strains of *B. typhosus* (Tilley and Schaffer 1928).

The same type of cut-off will be observed in cases of heterogeneous equilibrium concerning homologous series. It will always occur if on ascending the series the rate of decrease of solubility is greater than the rate of decrease of the distribution coefficient aqueous phase/non-aqueous phase. Thus the distribution coefficient between solution and surface which is an expression of surface tension lowering decreases by the ratio 3:3 for each member of a homologous series (Fraube's rule). The solubility decreases by the ratio 4. Therefore a point of maximum capillary activity will be reached on ascending a series. This is the case as shown by Clark's measurements of the surface tensions of aqueous alcohol solutions (Clark 1933). The position of the maximum will vary according to the value of the surface tension lowering taken for comparison. Adsorption on a solid surface will also show the same phenomenon. An example is supplied by Schilow and Nekrassow (1927), who found that in adsorption on charcoal from aqueous solutions the increase of adsorption corresponding to the addition of methylene groups exhibited a maximum value for the middle members—in their examples members with 5 or 6 carbon atoms.

Table V illustrates the use of the activity function in comparing the isonarcotic concentrations of substances of varied chemical composition applied in the vapour or gaseous state, as determined by Meyer and Hemmi (1935).

Narcosis is generally agreed to be due to a physical action of the molecule and not to a chemical reaction. The narcotic concentrations in Table V are equilibrium concentrations. Hence the activity function can be used to express the results. It is seen from Table V that though the isonarcotic concentrations of the different substances in air exhibit a wide range of value, the corresponding activities vary through a much more restricted

range. Meyer and Hemmi point out that the concentrations of these substances in olive oil solutions in equilibrium with the isonarcotic vapour concentrations also vary through a relatively restricted range (0.04–0.10). This is regarded as a support for the lipoid theory of narcosis. In effect, taken in conjunction with Table V, all it means is that the substances

TABLE V. ISONARCOTIC CONCENTRATIONS OF GASES AND VAPOURS FOR MICE AT 37° C

Substance	Saturation pressure at 37° C ( $p_s$ ) mm.	Narcotic conc. % by volume	Activity $p_i/p_s$
Nitrous oxide	59,300	100	0.01
Acetylene	51,700	65	0.01
Methyl ether	6,100	12	0.02
Methyl chloride	5,900	14	0.01
Ethylene oxide	1,900	5.8	0.02
Ethyl chloride	1,780	5.0	0.02
Diethyl ether	830	3.4	0.03
Methylal	630	2.8	0.03
Ethyl bromide	725	1.9	0.02
Dimethylacetal	288	1.9	0.05
Diethylformal	110	1.0	0.07
Dichloroethylene	450	0.95	0.02
Carbon disulphide	560	1.1	0.02
Chloroform	324	0.5	0.01

studied, when in dilute solution in olive oil, do not depart extremely from the behaviour expected by Raoult's law. Their observation while not inconsistent with the lipoid theory of narcosis in no way supports it. Table V indicates that narcosis might equally well be ascribed to adsorption on certain cell structures and that different substances produce equal depths of narcosis at adsorption potential values which lie within a relatively narrow range. The differences in the activity values are to be ascribed to differences in chemical constitution.

In Table VI the activities of various organic substances equi-toxic to *B. typhosus* have been calculated from the data of Tilley and Schaffer (1926, 1927, 1928). Again, though there are differences in activity, the range is restricted. Since it has already been shown that the alcohols act primarily physically upon this organism, and since the activities of the other substances of distinctly different constitution are of the same order of magnitude as the alcohol activities, it is a plausible assumption that they also act by a physical mechanism.

This argument is strengthened by a consideration of Table VII. Here, so far as the known vapour pressure data allow, the relative humidities

(activities) of the toxic concentrations of organic vapours to wireworms determined by Tattersfield and Roberts (1920) have been calculated. Fourteen substances of unknown vapour pressure have been omitted from the table. Twenty-seven other substances which were completely non-toxic to wireworms are also listed by Tattersfield and Roberts. These are all substances of high molecular weight and low vapour pressure. Their lack of toxicity is to be ascribed to the same type of effect as illustrated in fig. 2.

TABLE VI. BACTERICIDAL CONCENTRATIONS AND ACTIVITIES OF ORGANIC SUBSTANCES IN SOLUTION

Substance	Molecular phenol coefficient	Bactericidal concentration mol./l. $S_t$	Solubility at 25° C mol./l. $S_0$	Activity or $S_t/S_0$
Phenol	1.0	0.097	0.90	0.11
<i>o</i> -Cresol	2.5	0.039	0.23	0.17
*Methyl-octyl alcohol	0.009-29.0	10.8-0.0034	$\infty$ -0.004	0.33-0.88
Propaldehyde	0.09	1.08	2.88	0.37
Thymol	45	0.0022	0.0057	0.38
Acetone	0.025	3.89	$\infty$	0.40
Methyl ethyl ketone	0.078	1.25	3.13	0.40
Aniline	0.56	0.17	0.40	0.44
Cyclohexanol	0.55	0.18	0.38	0.47
Resorcinol	0.32	3.09	6.08	0.54
Methyl propyl ketone	0.25	0.39	0.70	0.56
Butyraldehyde	0.25	0.39	0.51	0.76

\* See Table II for individual values.

It will be seen that the substances can be arranged in two distinct classes on the basis of their toxic activities. There are those which are effective at very low values of  $p_t/p_s$  and those toxic in a range from 0.1 to saturation. The latter class are as a whole inert and unreactive substances of the typical narcotic type. If the substances are classed on the basis of their toxic concentration, the composition of groups of high, medium and low toxicity would be quite different from the above. Inert substances such as iodobenzene and bromoform would be classified along with highly toxic and reactive substances like hydrocyanic acid and ammonia. These latter substances are to be regarded as chemically toxic and the use of the activity index in such cases corresponds to no physico-chemical reality, since the toxic effect is not determined by an equilibrium concentration, but by the number of molecules reaching the seat of toxic action.

Consideration of Tables IV-VI leads to the conclusion that, in cases of physical toxicity or of narcosis, in order to exert a specified action on a

TABLE VII. INSECTICIDAL CONCENTRATIONS AND ACTIVITIES  
OF ORGANIC VAPOURS

Substance	Toxic concentration millionths g. mol. per l. lethal in 1000 min. at 15° C	Vapour pressure at 15° C mm. $p_s$	Relative humidity of toxic concentration $p/p_s$
Benzyl chloride	4	0.36	0.2
Chlorpierin	2	13.82	0.003
Monomethylanilino	3.7	0.22	0.3
Dimethylanilino	6.6	0.28	0.4
Phenol	10.6	0.15	Sat.
Hydrocyanic acid	20	500	0.0007
Ammonia	23	5450	0.00008
Monomethylamine	24	1850	0.00023
Dimethylamine	22	1090	0.00035
Ethylamine	22	720	0.0004
Nitrobenzene	24	0.08	Sat.
Aniline	27	0.28	Sat.
Iodobenzene	50	0.65	Sat.
o-Dichlorobenzene	70	0.77	Sat.
Pyridine	76	10.4	0.1
Bromoform	94	3.2	0.5
Bromobenzene	96	2.4	0.7
Monochlorotoluene	120	2.4	0.9
Tetrachloroethane	141	4.2	0.6
Chlorobenzene	200	6.8	0.5
p-Xylene	230	7.0	0.6
m-Xylene	230	7.0	0.6
Toluene	420	17.0	0.4
Carbon disulphide	526	245	0.04
Nitromethane	710	23	0.6
Benzene	775	58	0.2
Heptane	800	27	0.5
Chloroform	1040	128	0.2
Carbon tetrachloride	1600	73	0.4
Trichloroethylene	1200	52	0.4
Hexane	3000	96	0.6
Dichloroethylene	3100	230	0.2
Pentane	16600	320	0.9

selected organism the concentration of the substance applied in solution or in the vapour form must first be adjusted to a value such that its thermodynamic potential lies within a relatively restricted range of possible values characteristic of the organism. Thereafter a much smaller adjustment determined, in the first place, by the chemical constitution of the substance is necessary to obtain the effect specified.

Phase or adsorption equilibria are the main determinants of toxic concentration in such cases. To elucidate the mechanism of the essential

biological reaction this must be allowed for, and can be allowed for, by using, instead of the concentration, the activity function as a toxic index.

There does not exist at present a sufficient number of accurate toxicity data, together with activity data, to enable any profitable discussion to be made of the differences in activity value, which, in this paper, are ascribed to chemical constitution. The most that can be said is that increasing molecular weight is correlated with an increasing value of the toxic activity.

By itself a thermodynamic function cannot, of course, yield much information on mechanism. It is impossible to tell from activity data alone whether the distribution equilibrium is between two homogeneous phases or between a homogeneous phase and a surface layer. But a study of the behaviour of the activity function in other cases of heterogeneous equilibria—adsorption and simple distribution—should enable a solution to be reached.

The separation of toxic action into two main classes—chemical and physical—and the use of activity function in evaluating data dealing with the latter type, introduces, it may be claimed, a great clarification into the field of toxicity studies. The generalization that in cases of physical toxicity the activities of the toxic substances lie within a relatively narrow range for any one organism, embraces in one statement many published generalizations of narrower scope. From it, for example, follow Richet's rule (1893) according to which the "toxicity" of a substance is roughly inversely proportional to its solubility, and the boiling point rule of Moore (1917) whereby the "toxicity" of insect fumigants increases with boiling point and consequently with decreasing vapour pressure. The parallelism between physiological action and oil-water distribution ratios is really a case of Richet's rule and thus comes within the activity generalization. The same may be said of the parallelism between physiological action and surface tension lowering. From the work of Fühner (1921), it is apparent that there is no intrinsic connexion between the two, and where a correlation does exist it is merely due to the fact that highly surface active substances are also usually very slightly soluble.

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## SUMMARY

From a review of published data it is concluded that two main classes of toxic action exist. In one class the effects are due to chemical reaction. In the other a physical mechanism is at work. Criteria are given whereby the two classes of toxic action may be distinguished. All substances can exert physiological effects by a physical mechanism though the physical action may be, and frequently is, masked by a specific chemical reaction.

Physiological effects due to a physical mechanism are generally measured when an equilibrium has been established between the concentration of the toxic substance in the phase, solution or vapour, in which it is applied and the concentration in the phase or surface layer which is the seat of toxic action. Since the chemical potential of the toxic substance must be the same in all phases partaking in the equilibrium, it is suggested that the toxicities of physically toxic substances should be compared, not by the values of the toxic concentration in the external solution or vapour, but by the values of the chemical potentials in these phases. Chemical potentials so determined are identical with the chemical potentials at the actual point of attack within the organism. By this procedure the disturbing effect of phase distribution is eliminated from the comparison of toxicities.

Using the activity function of G. N. Lewis as the chemical potential, it is found that, when chemical reaction is absent, though diverse chemical compounds exert the same toxic effect on a given organism at widely different concentrations, the activities corresponding to these concentrations lie within a relatively narrow range. The differences in activity within this range are ascribed to the effect of chemical constitution.

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# Some factors producing individual differences in dark adaptation

BY L. R. PHILLIPS

*From the Department of Psychology, University College, London*

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Since Aubert's (1864) original work, many attempts have been made to describe or to classify individual variations in dark adaptation. Piper (1903) and later Wölfflin (1905) distinguished two main classes, one showing a rapid initial rise in sensitivity, with a high final maximum, and the other a slower increase, with a lower final value. Lohmann (1907) showed that dark-adaptation curves obtained with the homatropinized and eserinated eye were very different and suggested that pupil diameter was the underlying factor. Best (1917) found two main types, one with a rapid initial increase in sensitivity, and the other with a slower increase, both reaching approximately the same final values. He nevertheless states that the final thresholds of normal observers differ by as much as 12 : 1. Cobb (1919) found threshold differences of 7.4 : 1 after 30 min. in the dark. Flugel (1921) also mentions two classes characterized by unusually slow, or unusually rapid, initial rises in sensitivity. Wynn Jones (1921) found considerable differences in observers (25 : 1 after 16 min. in the dark), dividing them into those whose dark adaptation was (a) poor at the beginning and at the end, (b) poor at the beginning and good at the end, (c) good at the beginning and good at the end, (d) good at the beginning and poor at the end. Matthey (1932) also grouped individuals into the same four types.

Gross deficiency in vitamin A undoubtedly produces marked differences in dark adaptation (see for instance Tansley 1939). Convincing data on this subject have been advanced by Fridericia and Holm (1925), Tansley (1931) and by Guilbert and Hart (1935), in animals. Recently Edmund and Clemmesen (1936) and others have claimed that a small deficiency even in apparently healthy individuals will produce some slowing in the process of dark adaptation. Excluding deficiency in vitamin A, no physiological factor is generally held to be responsible for individual variations in dark adaptation, nor is any real division into types recognized.

The present experiments were designed to determine the influence which the diameter of the pupil, colouring, visual acuity and age might exercise on the dark adaptation of individuals, after variations due to deficiency in vitamin A had been as nearly as possible eliminated.

### APPARATUS

A description of most parts of the apparatus has been given in a previous paper (Lythgoe and Phillips 1938).

Briefly it consisted of a white octagonal chamber in which the observer sat facing an opal glass test patch. During light adaptation the chamber was capable of being evenly illuminated over a wide range of values, with a maximum, as measured by a lumeter, of 130 equiv. ft. c. The test-patch illuminations were projected from a room behind the observer by means of a lens, through a shutter which regulated the intermittent stimuli. The illuminations were provided by calibrated and standardized headlamp bulbs working under known conditions of voltage, whilst changes in illumination were provided both by moving these lamps on a 3 m. optical bench, and by the interposition of suitable diaphragms between the components of the projecting lens. Elaborate precautions were taken for correcting candle power changes in the lamps due to ageing. Calibration of the test-patch illuminations was by a substitution method, using a Weston photronic cell in series with a sensitive galvanometer as photometer, and expressing illuminations in terms of the candle power of a lamp standardized by the National Physical Laboratory. Correcting for the reflexion factor of the test patch, the total range of test-patch brightnesses was found to be from  $5.6 \times 10^{-3}$  to  $2.2 \times 10^{-7}$  equiv. ft.c.

The test patch was circular, and subtended a visual angle of  $12\frac{1}{2}^\circ$ . The stimuli occurred every 2.5 sec. for a duration of 0.2 sec. The red (Wratten no. 25) fixation spot was projected to the centre of the test patch, and its brightness could be varied by the observer. Fixation was thus central, and observation circumfoveal. The onset of darkness, the responses of the observer, the time of each flash, and 5 sec. time signals were recorded by a three-pen marker on a moving band of paper.

The additional apparatus necessary for pupil photography was the same as that used by Lythgoe (1932) except that the eye was illuminated by an infra-red flash and infra-red plates were used. Fixation spots were arranged so that the accommodation was exactly the same for all observers. The exposure both during light adaptation and in the dark was  $\frac{1}{8}$  sec., this being short enough to eliminate pupil movements. The negatives were of normal density and their reduction was 0.503. The pupil diameter was measured directly from them by means of a travelling microscope. Since relative size only was required, the apparent pupil diameter uncorrected for corneal refraction was used in the calculations.

### EXPERIMENTAL PROCEDURE

The experiments were divided into two parts. In the first, male observers, of much the same age, and approximately emmetropic, were dosed with vitamin A for some days previous to the determination of dark adaptation. In the second, observers of both sexes, of widely different ages, were tested

without previous dosage, but with chances of serious vitamin A deficiency reduced by the choice of suitable observers.

In the first group, the influence of pupil diameter, general colouring and visual acuity were investigated, after possible variations due to age and vitamin A content had been eliminated. The observers were male medical students of approximately the same age, and each had received regular dosage amounting to 100,000 units of vitamin A spread over a period of 6 days previous to his examination.

Previous study of day to day variations in dark adaptation had shown that the effects of exposure to strong daylight upon subsequent dark adaptation were far reaching, especially if observers had experienced it a short while before their entry into the light adaptation chamber. For this reason the examinations were conducted after dark in the evenings of the winter months, from December to March. In a similar study it was found that, with the method of testing employed, practice effects were small. Any effects which might be present due to this cause were small compared with day to day variations of the observer, and quite insignificant when compared with the differences in dark adaptation found in different individuals.

Upon arrival, the observer was given a short ophthalmological examination. The fundus was examined so as to exclude pathological abnormalities, and in addition an estimate was made of the quantity of retinal pigment. The latter was done both by noting the general colour of the fundus (slate, bright red, etc.) and by noting whether the retinal pigment was scanty enough to render visible the choroidal vessels in the peripheral retina. Visual acuity was determined for the two eyes separately, and also together. Snellen's test types at 6 m. were used, illuminated by 4.4 ft.c. Using both eyes, with glasses when these were worn for the test, thirty-eight out of the forty observers had vision better than 6/6 (75 % of letters), one had vision of 6/12 (all), and another 6/9 (50 %). A rough estimate of the observer's refractive error was made by the subjective method. Apart from observer no. 20 who had an error of  $-4.5$  D. Sph. (L.E.) and  $-0.5$  D. Sph. (R.E.), the refractive error of no observer in this group exceeded  $+1.25$  D. or  $-1$  D. in any meridian for either eye. In addition, tests were made for heterophoria, stereoscopic fusion, colour blindness and colour anomalies. Estimates were made of the iris colouring and the general colouring or pigmentation. Finally, the observer answered a questionnaire calculated to betray difficulties of night vision, abnormalities of diet, and exceptional medical or ophthalmological histories.

The preliminary series of examinations occupied half an hour. During this period care was taken that the conditions of illumination were as nearly

as possible the same for all observers. The observer then entered the light adaptation chamber. For 5 min. the subsequent procedure was explained to him, the brightness of the walls being 17 equiv. ft.c. during this period. After a trial run, lasting 5 min., which ensured that the observer fully understood his instructions, the actual run was taken. This consisted of a light adaptation period of 8 min. at 86 equiv. ft.c., a pupil photograph being taken at the end of the second minute. The observer was then plunged into darkness, whilst a predetermined illumination was projected on the test patch for 0.2 sec. every 2.5 sec. As soon as he recorded that he had seen a flash, the test-patch illumination was reduced to a second fixed value, and this procedure repeated until, after 15–20 min. in the dark, the observer had seen a final arbitrary illumination. Finally, whilst still in darkness, the pupil was photographed by infra red light. Previous study had shown that the observers gave smoother and more reliable curves when they were instructed to signal only when they were quite sure that they had seen a flash. The reliability of the observer was tested during the trial run by a simple system of right and wrong responses. This was the only method used of checking results. Observation and light adaptation were binocular throughout.

The procedure adopted in the examination of the second group was identical with that already described, except that the preliminary dosage with vitamin A and the ophthalmoscopic examination were omitted.

For convenience of working arbitrary scales have been used in the general analysis of the data which follows. Pupil diameters have been expressed in  $\frac{1}{200}$  mm., while the scale of general colouring was an arbitrary one of from 1 to 10, maximum darkness or pigmentation being 10. An arbitrary scale of visual acuity was used based on the performance with the Snellen types. Thus 6/3 (all letters read) scored 20, 6/3 (75 % letters read) scored 19, 6/3 (25 %) scored 18, 6/4 (all) scored 17, and so on to 6/12 (75 %) which scored 1. The following scale was used in scoring the density of the retinal pigment. A slate retina (such as is found in dark races) scored 5, a slatish retina 4, pale red 3, red 2, and bright red 1. The opacity of the retina was recorded by giving 4 points to a retina through which no choroidal vessels could be seen in the periphery, 3 points for choroidal vessels just seen, 2 for choroidal vessels easily seen, and 1 for choroidal vessels very easily seen.

For brevity, the term "perception time" has been used in the text to represent the time elapsing from the onset of darkness to the moment of perception of any one test-patch brightness.

## RESULTS

The tests were completed by the forty-six observers who comprised the first group. Of these the results of six were excluded, three because they were unable to fulfil the criterion of reliability (i.e. they were apt to respond when no flash was present), and three because their curves (fig. 1) were grossly abnormal. The remaining forty observers were male, of western European extraction, of average age 20 yr. 8 mo., aged from 19 to 22 years with the exception of four, who were aged 28, 28, 27 and 26 years.

The curves of all forty observers are shown in figs. 2 and 2A. The curve of observer 18 is representative of twenty-three of the forty curves. It shows the smooth rapid fall in the threshold during the first 2 or 3 min., attributed to changes in cone sensitivity, and the subsequent gradual fall in the threshold to the end of the run, attributed to the rods (Kohlrausch 1922). When plotted on a linear time scale the relative slopes of the two portions of the curves are more clearly shown, and the transition becomes more abrupt. In thirteen of the forty curves, an irregularity of the curve occurring between 50 and 250 sec. is shown to a greater or less degree. This irregularity is well seen in observer 36. It may be a true effect indicating that dark adaptation is not a twofold process, or it may be due to a contraction followed by a dilatation of the pupil such as has been noticed by Crawford (1936). A similar disturbance is often noticed when a dark adaptation run is taken too soon after the introduction of a mydriatic into the eye. The curve of observer 7 is typical of the remaining four curves, in that it demonstrates marked oscillations which might be due to an extension of the disturbance shown by observer 36, but is more probably due to poorness of observation. Such oscillations have been noticed by other workers, notably by Aubert (1864) and Achmatov (1926).

The greatest observed threshold difference, after 15 min. in the dark, was 10:1. This value may be compared with the ratios given in the introduction, although differences exist in the conditions of the experiment, and the area and position of the retina excited.

*The influence of pupil diameter in the light.* Let  $X(L1)$  and  $X(D1)$  represent the pupil diameter in the light and in the dark respectively for observer 1, and  $y(A1)$  to  $y(Q1)$  the times at which the same observer saw the test-patch brightnesses  $A$  to  $Q$ . For forty observers we have

$$\begin{array}{cccccc} X(L1), & X(D1), & y(A1), & y(B1), & \dots, & y(Q1), \\ X(L2), & X(D2), & y(A2), & y(B2), & \dots, & y(Q2), \\ \dots & \dots & \dots & \dots & \dots & \dots \\ \dots & \dots & \dots & \dots & \dots & \dots \\ X(L40), & X(D40), & y(A40), & y(B40), & \dots, & y(Q40). \end{array}$$

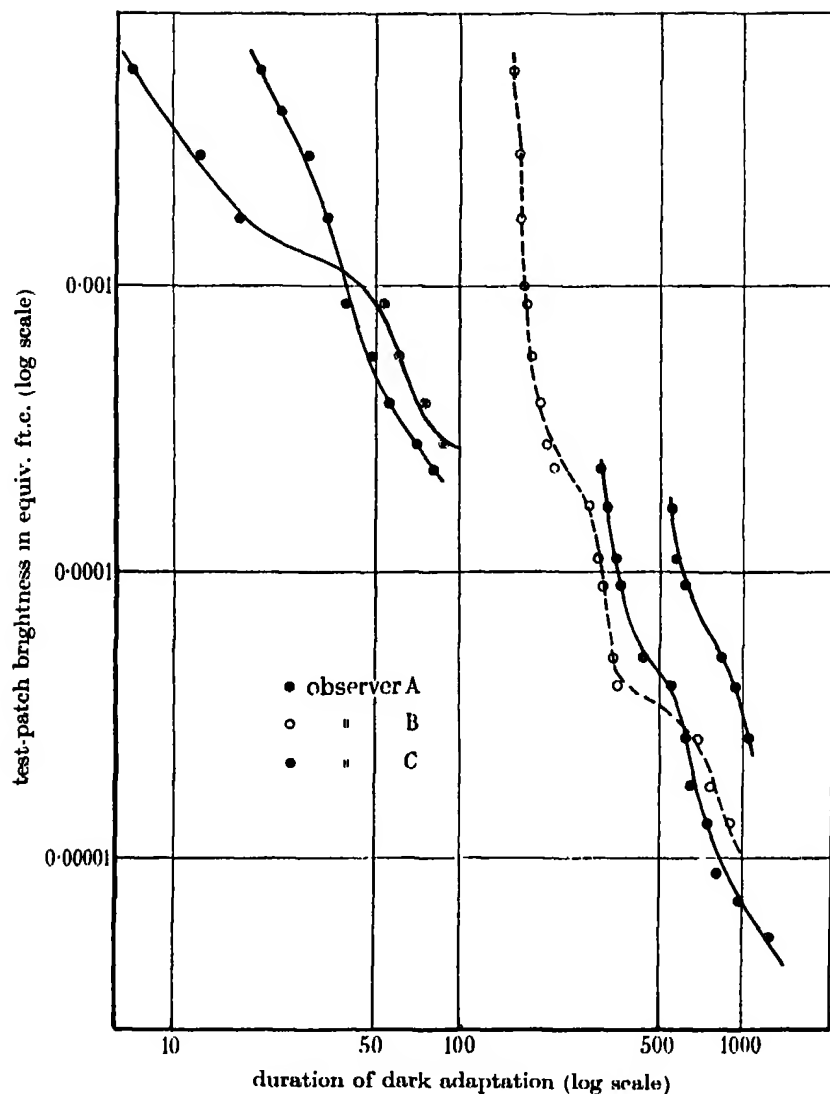
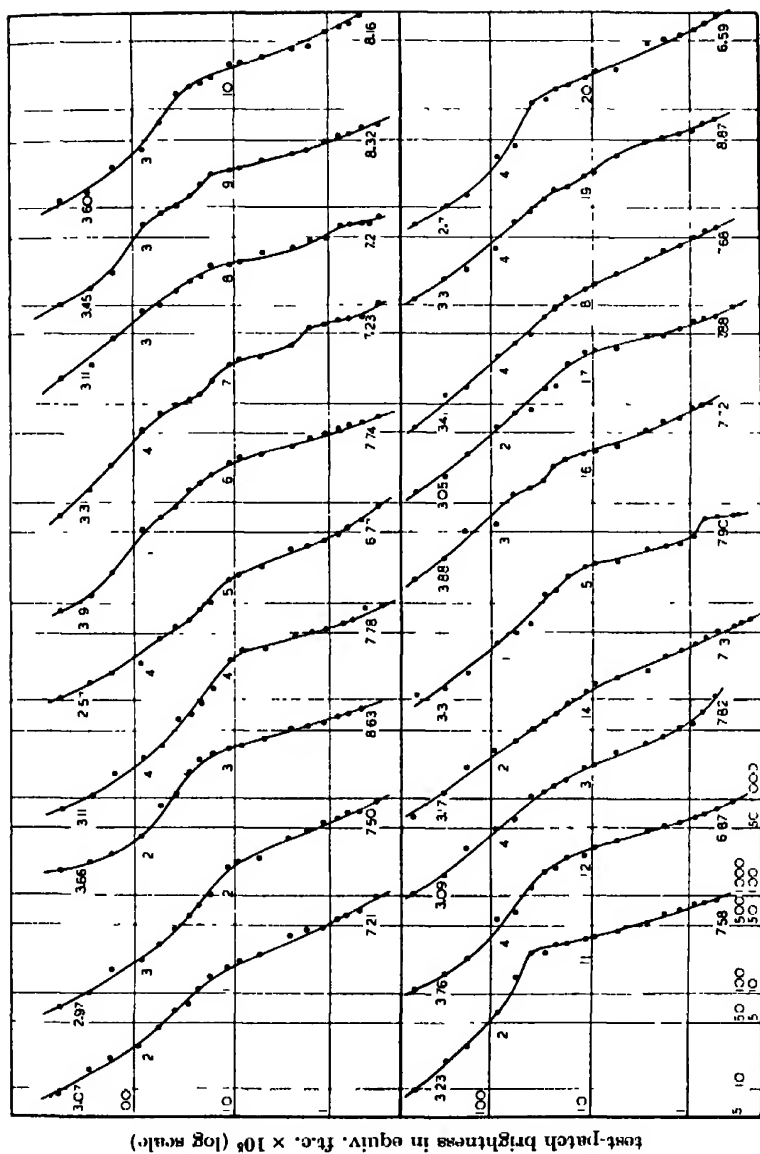


FIG. 1. The three grossly abnormal curves of dark adaptation. Observer A. Aged 20. Measles, 1922. Diphtheria, 1923. Diet normal. Non-smoker. Emmetropic. Vision 6/5 (75%), each eye. Wears +1.0 D. Sph. each eye for reading. Fundi normal. Observer B. Aged 21. Measles. Diphtheria (doubtful). Severe general infection, China. Trachoma, China, 1921. Boils. C/o dazzling in sunlight. Diet normal. Non-smoker. Very slight myopia (-0.75 D. Cyl. vertical, each eye). R.E. reads 6/6 (25%). L.E. reads 6/9 (all). Conjunctivae injected, pupils small, fundi normal. Observer C. Aged 21. Usual childish diseases. Diphtheria, 1921. Diet normal. Smoker, 100 cigarettes a week. Emmetropic. Vision 6/3 (25%), each eye. Colouring fair. Fundi normal.



duration of dark adaptation in sec. (log scale)

FIG. 2. Dark adaptation curves of twenty observers. First group. The numbers to the left of each curve, reading from top to bottom, give the apparent pupil diameter in the light (in mm., uncorrected for corneal refraction), the measure of the retinal pigment (as determined by the visibility of the choroidal vessels), the number of the observer, and the pupil diameter in the dark. The abscissae of successive curves are moved ten times to the right, i.e. the same abscissa will be 10 sec. for the top curve, 100 sec. for the middle curve and 1000 sec. for the bottom curve.





The table on p. 409 clearly permits the correlation of any one column with any other. Thus we may correlate  $X(L1) \dots X(L40)$  with  $y(A1) \dots y(A40)$  which will give us the correlation of pupil diameter in the light with perception time for test-patch brightness  $A$ . This operation may be repeated for each of the seventeen test-patch brightnesses, giving seventeen correlation coefficients which will show the degree of the influence of pupil diameter in the light during the first 20 min. of dark adaptation. All seventeen coefficients so obtained are positive (Table I).

TABLE I. CORRELATION COEFFICIENTS SHOWING THE INFLUENCE OF PUPIL DIAMETER IN THE LIGHT ON PERCEPTION TIME FOR SEVENTEEN TEST-PATCH BRIGHTNESSES. TEST-PATCH BRIGHTNESSES GIVEN IN  $\text{LOG}_{10}$  EQUIV. FT.C. + 10 UNITS

Test-patch brightness	7.75	7.45	7.23	6.93	6.75	6.59	6.45	6.35	6.23
Correlation coefficient	0.30	0.45	0.53	0.45	0.48	0.46	0.50	0.53	0.52
Test-patch brightness	6.05	5.95	5.72	5.42	5.23	5.09	4.95	4.85	
Correlation coefficient	0.62	0.41	0.35	0.30	0.32	0.35	0.38	0.33	

Using the customary technique for the significance of correlation coefficients of small samples (Fisher 1936, p. 195), the borderline value for this number of terms for the 5% level of significance (the usual criterion of reasonable certainty) is 0.31, whilst for the highly significant 1% level it is 0.40.\* Of the seventeen coefficients, fifteen are significant, whilst of these ten exceed the highly significant 1% level. The remaining two values are sufficiently high to allow us to assume their probable significance.

We must therefore conclude that the pupil diameter during light adaptation has a significant influence on the subsequent course of dark adaptation, even at the end of the period examined. The positive nature of the coefficients shows that with a small pupil dark adaptation is rapid, a result

\* To those unaccustomed to the expression of the significance of the correlation coefficients in this way, it should be explained that the 5 and 1% levels are expressions of  $P$ , the probability that the absolute magnitude of an observed value, regardless of sign, will exceed a specified absolute magnitude. The customary criterion of reasonable certainty is that this probability shall not exceed 0.05, usually termed the 5% level, which implies that the odds against the fortuitous appearance of a coefficient of such magnitude in the data are 20:1 against, with corresponding odds of 100:1 against for the 1% level. It clearly follows that the appearance of a large proportion of significant coefficients from the data considerably enhances the significance of the result.

in accordance with those of other workers on the retardation of dark adaptation with increased light adaptation (Charpentier 1886; Hecht and Haig 1936, etc.).

This result may be summarized by determining the significance of the regression of time on pupil diameter in the light for the whole of the data, i.e. for all seventeen test-patch brightnesses. For if we regard pupil diameter in the light as the independent, and perception time as the dependent variate, then it is possible to determine the regression of time on pupil diameter in the light for any one brightness, and, by an analysis of variance, to determine its significance.

Determining the significance of the regression for test-patch brightness 7.75, we have:

Source of variation	Degrees of freedom	Sum of squares	Mean square	$\frac{1}{2} \log_e$
Regression	1	35.67	35.67	1.787
Remainder	38	252.43	6.64	0.946
Total	39	288.1	$z = 0.84$	

For the 5% level of significance  $z = 0.70$  and for the 1% level  $z = 1.00$ . Since our value of  $z$  lies above the 5% level, our regression is of undoubted significance.

This analysis might be repeated for the remaining sixteen test-patch brightnesses. It is simpler to combine the data for all the test-patch brightnesses and hence to obtain one regression whose significance will summarize the influence of pupil diameter in the light over the whole period of dark adaptation examined. The analysis of variance so obtained is:

Source of variation	Degrees of freedom	Sum of squares	Mean square	$\frac{1}{2} \log_e$
Regression	1	55797	55797	5.4647
Remainder	646	4616344	7146	4.4371
Total	647	4672141	$z = 1.0276$	

For the 5% level  $z = 0.68$  and for the 1% level  $z = 0.95$ . The value of  $z$  lies above the 1% level, and the regression over the whole data must therefore be considered to be very highly significant. This result summarizes and confirms the results obtained by the examination of the correlation coefficients and points to the undoubted influence of pupil diameter in the light upon subsequent dark adaptation.

*The influence of pupil diameter in the dark.* The same procedure was adopted as that already described for pupil diameter in the light except

that correlations were made for only six test-patch brightnesses distributed over the 20 min. of dark adaptation tested. The resulting correlation coefficients (Table II) are not significant, and a study of the regression over the whole of the data also yields a negative result.

TABLE II. CORRELATION OF PUPIL DIAMETER  
IN THE DARK WITH PERCEPTION TIME

Test-patch brightness	7.75	7.23	6.45	5.95	5.23	4.85
Uncorrected coefficient	0.19	0.07	0.06	0.00	-0.07	-0.09
Partial correlation	0.08	-0.18	-0.18	-0.19	-0.23	-0.25
Corrected for pupil diameter in light	-0.11	-0.17	-0.24	-0.33	-0.35	-0.51

We should certainly expect a large pupil diameter to improve vision in the dark. The clue to this unexpected result is obtained when we correlate pupil diameter in the light with pupil diameter in the dark for the forty observers. The resulting coefficient has the highly significant value 0.39, which implies that an observer with a small pupil diameter in the light will, in general, have a comparatively small pupil diameter in the dark. Let us consider an observer whose pupil diameter in the light is comparatively small. We should expect his curve of dark adaptation to be early, but since his pupil diameter in the dark is probably smaller than normal this would tend to make his curve late. The influence of pupil diameter in the light will tend, therefore, to mask the effect of pupil diameter in the dark.

The effect of pupil diameter in the light may be removed by partial correlation using the equation

$$r_{12.3} = \frac{r_{12} - r_{13} \cdot r_{23}}{\sqrt{\{(1 - r_{13}^2)(1 - r_{23}^2)\}}},$$

where  $r_{12.3}$  is the correlation of pupil diameter in the dark with perception time but with pupil diameter in the light eliminated,  $r_{12}$  is the correlation coefficient of pupil diameter in the dark with time,  $r_{13}$  is the correlation coefficient of pupil diameter in the light with pupil diameter in the dark, and  $r_{23}$  the same for pupil diameter in the light with time. Individually, the coefficients are not significant (Table II), but the odds against five negative coefficients of such magnitude occurring together by chance are very great.

This result is substantiated by an empirical method of approach. This consisted in subjecting an observer to three different degrees of light

adaptation, measuring his pupil diameter under these conditions. From the position of the three subsequent dark-adaptation curves, an estimate can be made of the displacement caused by a change of one unit of retinal illumination (i.e. one photon) in the previous light adaptation. Using this estimate, all the perception times of the various observers were corrected so as to produce times which would have been recorded had every observer possessed the same arbitrary pupil diameter in the light. The corrected times were then correlated with pupil diameter in the dark, so obtaining the true influence of pupil diameter in the dark on the data.

To this end a trained observer completed eighteen dark-adaptation curves in six sittings under three conditions of light adaptation, the three conditions being combined in every possible arrangement in the six runs. This gave three mean dark-adaptation runs for the three light-adaptation intensities (the great advantage of the present method of recording dark adaptation is that it allows the times for the perception of any one test-patch brightness to be averaged). For the three mean pupil diameters of 4.51, 3.62 and 3.46 mm., the three corresponding retinal illuminations were 958, 2116 and 2336 photons.

TABLE III. OBSERVED AND CALCULATED VALUES OF PERCEPTION TIMES (SEC.) FOR PRECEDING RETINAL ILLUMINATION OF 2336 PHOTONS FOR 8 MIN.

Test-patch brightnesses	7.8	7.4	7.0	6.6	6.2	5.8	5.4
Times at which brightnesses were seen:							
Obs.	9.3	18	42	81	144	245	371
Calc.	9.5	18	35	74	148	240	436

The three dark-adaptation curves so obtained were approximately parallel. For the curves taken with the two lower light adaptations the differences in log time at brightnesses 7.8, 7.4, 7.0, 6.6, 6.2 and 5.8 were 0.23, 0.22, 0.24, 0.25, 0.20 and 0.21 log units respectively. Thus a difference of 1158 photons produced a mean displacement of the dark-adaptation curve of 0.23 units in log time. In other words, an increase of 1 photon makes the perception times throughout the curve 0.05% longer. Using this rule, the expected displacement for a difference of 1378 photons (the difference between the extreme retinal illuminations) has been calculated, and compared with the actual values taken from the third, or latest curve. The agreement is good for all brightness values except the last (Table III), where the curves are no longer parallel. This result has been obtained from only one observer, and it may not be true for all

observers. Its application is also limited to light adaptation for a period of 8 min.

Let us suppose that an observer's pupil diameter in the light is  $A$  mm., and that we wish to displace the curve to correspond to an arbitrary pupil diameter in the light of  $B$  mm. From the definition of a photon, it follows that the retinal illumination due to a brightness of  $P$  equiv. ft.c. and a pupil diameter of  $A$  mm. is  $2.69 PA^2$  photons. Then since one photon produces an approximate displacement of the dark-adaptation curve of  $0.00020$  log unit, the required displacement will be  $0.00020 \times 2.69 P(B^2 - A^2)$  log units. This logarithm, when added to the logarithm of the actual perception time, gives the logarithm of the corrected time, i.e. the hypothetical time which the observer would have recorded had his pupil diameter been  $B$  mm. For example, after a light adaptation of 130 equiv. ft.c., a change in a pupil diameter in the light from 2.5 to 3.0 mm. would require an addition of 0.19 to the logarithm of each perception time, or, more simply, each perception time would require to be increased by 54 %.

These corrections were applied to the perception times of all the observers, the arbitrary value of  $B$  being 3.23 mm., the mean of the pupil diameters in the light of the forty observers. The corrected times were correlated with pupil diameter in the dark (Table II last line).

Applying the criteria of 0.31 and 0.40 for the 5 and 1 % levels of significance respectively, we find the last three coefficients significant, with the last coefficient exceeding the 1 % level. This confirms the results found by partial correlation. We must therefore conclude that there is undoubted evidence that pupil diameter in the dark is a factor influencing the course of dark adaptation, but that its influence is in general masked by the indirect influence of pupil diameter in the light.

It has been found that a simpler correction (which might, for instance, be used at a clinical examination) would be to multiply each perception time by  $B^2/A^2$ . Though this mode of correction has no theoretical justification, it agrees remarkably with the more elaborate correction already proposed.

Although the suggestion is somewhat hypothetical, it is possible that differences in the pupil diameters in the light and in the dark explain, at least in part, the types of observer found by Wynn Jones and Matthey. For we can group observers into those with

- (a) pupils large in the light and small in the dark,
- (b) pupils large in the light and large in the dark,
- (c) pupils small in the light and large in the dark,
- (d) pupils small in the light and small in the dark.

These would clearly produce four classes of curve, in which dark adaptation would be

- (a) poor at the beginning and poor at the end,
- (b) poor at the beginning and good at the end,
- (c) good at the beginning and good at the end,
- (d) good at the beginning and poor at the end.

Because a large pupil in the light is in general associated with a large pupil in the dark, and a small pupil in the light with a small pupil in the dark, we should expect classes (b) and (d) to predominate. These might easily be the two types noticed by Flugel and others.

That examples of the latter two types predominate in the data is shown by plotting the mean curves of ten early, and ten late observers, and comparing them with the mean curves of the same observers after the perception times have been corrected for differences of pupil diameter in the light (fig. 3). It will be noticed that the corrected curves cross one another. This is to be expected since the ten early curves will be those of observers having small pupil diameters in the dark (class d), and the second portions of the curves will therefore tend to be late. The reverse argument applies to the ten late curves (class b). When the second portions of the two corrected curves are further corrected on the assumption that the retinal illumination in the dark is proportional to pupil area, the latter parts of the curves are almost coincident. We can therefore conclude that for the later parts of the curves practically all individual variations can be explained in terms of pupil diameter, provided the observers are all of approximately the same age. The assumption that retinal illumination is proportional to pupil area is justified only when dealing with low illuminations (Stiles 1937). At higher illuminations (i.e. for cone vision) this is not a valid correction (Stiles and Crawford 1933). No pupil corrections could adequately explain the residual individual differences of the first 2 min. of dark adaptation.

The values of the coefficients correlating pupil diameter in the dark with perception time increase progressively with time in the dark (Table II). The unexplained individual variations during the early part of dark adaptation probably account for this progressive increase since they mask the effects of pupil diameter and make the early coefficients smaller than they would otherwise be.

Dr Katharine Tansley, to whom my thanks are due, has produced nineteen references from the literature on hemeralopia in which there is specific mention of pupillary abnormality accompanying the condition. Fifteen papers mention that dilated pupils accompanied the condition in

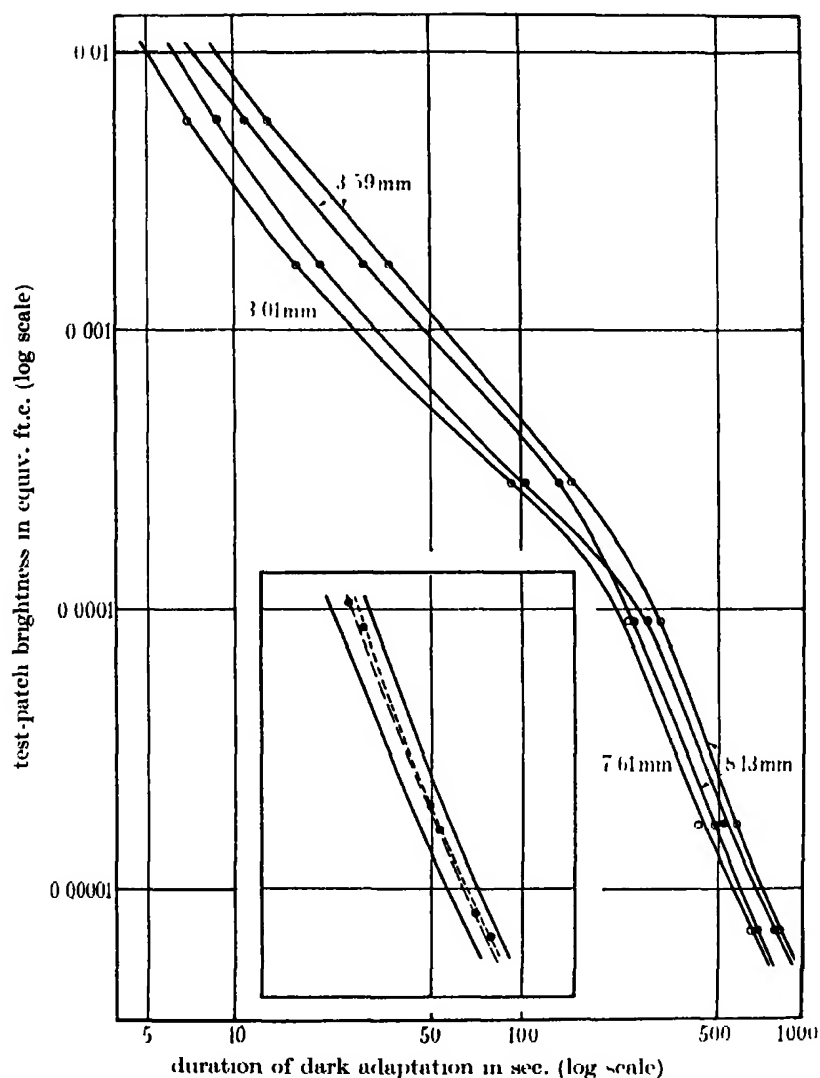


FIG. 3. Mean dark-adaptation curves of ten early and ten late observers, before and after correction for pupil diameter in the light. The appropriate mean diameters of the pupil in the light and in the dark are shown on the curves.  $\circ$ — $\circ$  Uncorrected curves.  $\odot$ — $\odot$  Corrected curves for observers with large pupils.  $\bullet$ — $\bullet$  Corrected curves for those with small pupils. Inset: Corrected and uncorrected curves after a further correction for pupil diameter in the dark (p. 417).

man, whilst two note it in swine. Of the remaining papers, one reports that dilated pupils were present in only a few cases, and the other reports a case of pupillary contraction. Unfortunately the condition was often associated with scurvy and other pathological conditions which make direct inference from the data difficult.

TABLE IV. CORRELATION COEFFICIENTS SHOWING THE INFLUENCE OF COLOURING AND VISUAL ACUITY UPON PERCEPTION TIME: FORTY OBSERVERS

Test-patch brightness	7.75	7.23	6.45	5.95	5.23	4.85
Colouring, correlation	0.15	0.09	0.03	-0.09	0.09	-0.01
Visual acuity, correlation	-0.04	0.05	0.02	-0.04	-0.10	-0.18

*The influence of colouring and visual acuity.* The methods adopted for the analysis of the influence of pupil diameter were applied to determine the influence of colouring and visual acuity. This produced six correlations for both factors. These are given in Table IV. None of these values approaches significance, the largest, -0.18, being one which might easily appear by chance in a table of this size. These correlations were not improved when the influence of differences in pupil diameter in the light were removed from the data by partial correlation or by the empirical method. It should be noted that the visual acuity values used in the correlations were those obtained under the conditions of the dark adaptation run. Thus, if the observer wore glasses in the dark, the appropriate visual acuity value, obtained with those glasses, was used.

*The influence of retinal pigmentation.* The measures of the colour of the fundus and the visibility of the choroidal vessels, obtained at the ophthalmoscopic examination, were correlated with the perception times for six selected test-patch brightnesses. All the coefficients are negative (Table V), whilst one exceeds the 5% and one the 1% level of significance. Thus, although not conclusive, there is some evidence that heavy pigmentation in the retina favours more rapid dark adaptation. Wölfflin (1905) arrived at a similar conclusion, being of the opinion that very fair persons adapted comparatively slowly. It is probable that the heavier pigmentation offers some protection to the photosensitive substances in the retina during the preliminary light adaptation. No appreciable correlation was found between the measures of pigment and the pupil diameter in the light and in the dark. The correlation coefficient relating the general colouring of the individual, and the visibility of the choroidal vessels, was 0.04. Thus, for the group examined, the colouring of the individual was no indication of the degree of pigmentation of his retina. The group, however, included



no very fair nor very dark persons. With such extremes one would expect corresponding variations in the retinal pigmentation, and, as was suggested by Wölfflin, the poor adaptation of his fair observers was probably due to a deficiency of intraocular pigment.

TABLE V. CORRELATION OF RETINAL PIGMENTATION WITH PERCEPTION TIME FOR SIX TEST-PATCH BRIGHTNESSES

Measure of pigment	Test-patch brightness					
	7.75	7.23	6.45	5.95	5.23	4.85
Colour of fundus	-0.07	-0.19	-0.13	-0.02	-0.02	-0.04
Visibility of choroidal vessels	-0.02	-0.22	-0.13	-0.31	-0.18	-0.17

The correlation of the colour of the fundus (slate, bright red, etc.) with perception time yielded six negative coefficients, none of which was significant (Table V). It is probable that this inconclusive result is due to the inaccuracies of the method used to estimate the colour.

*The influence of age on dark adaptation.* For this investigation twenty-six observers of both sexes were chosen, whose ages ranged from 17 to 70 yr. with an average age of 30 yr. 6 mo. The procedure was identical with that already described for the first group, except that the previous dosage with vitamin A and the ophthalmoscopic examination were omitted. The social position and diet of these observers were such as to make unlikely the presence of serious deficiency of vitamin A.

The ages of the twenty-six observers were correlated with their perception times for four test-patch brightnesses. The resulting correlation coefficients are shown in Table VI. Applying the criterion of significance, all these coefficients exceed the highly significant 1 % level (0.49). We must therefore conclude that there is undoubted evidence that age has a considerable effect on dark adaptation. The positive nature of the coefficients points, as one would expect, to a slowing of the process of dark adaptation with age. Most previous workers like Wölfflin (1905), although they were of the opinion that the process of dark adaptation was slowed with age, did not obtain such a definite result as has been found in these experiments.

TABLE VI. CORRELATION OF AGE WITH PERCEPTION TIME.  
SECOND GROUP OF TWENTY-SIX OBSERVERS

Test-patch brightness	7.75	7.23	6.45	5.23
Correlation coefficient	0.81	0.63	0.57	0.57

A further correlation in this group is that of pupil diameter in the light with age. The coefficient, -0.58, exceeds the 1 % level. The negative value

points to the decrease of pupil diameter with age, a relation already well known. The correlation is interesting in that it shows that if the data of the second group were corrected for the effects of different pupil diameters in the light, the tendency would be still further to retard the curves of the older observers, with a probable increase in the already high correlation coefficients of age with perception time.

The mean pupil diameter in the dark of the ten oldest observers was 6.86 mm., a value significantly lower than the mean of the group (7.44 mm.). Some part of the retardation of dark adaptation with age is therefore due to a small pupil diameter in the dark. The remainder must be assumed to be due to changes in the retina with increasing age.

In conclusion, I wish to thank Dr R. J. Lythgoe for the ophthalmoscopic, visual acuity and refractive error examinations. Without his help and ungrudging co-operation this work would not have been possible. In addition my thanks are due to W. L. Stevens, Esq., of the Galton Laboratory, for suggestions respecting the statistical treatment of the data.

#### SUMMARY

1. The course of dark adaptation was examined over a period of 20 min. for a white intermittent stimulus of 0.2 sec., centrally fixated, and subtending a visual angle of  $12\frac{1}{2}^{\circ}$ . All observers were selected so as to exclude both gross optical and other abnormalities.

2. In the first group of observers there were forty males of much the same age (av. 20 yr. 8 mo.), possible deficiency in vitamin A being eliminated by systematic dosage. Various ocular characters were correlated each in turn with the times taken from the onset of darkness to see each of the selected brightnesses of the test patch (for brevity these times are referred to as "perception times").

3. Correlation of pupil diameter in the light with perception time yielded fifteen significant positive correlation coefficients for seventeen test-patch brightnesses. This result was confirmed by showing the significance of the regression of time on pupil diameter in the light for the whole of the data. A comparatively large pupil in the light, therefore, produces a retardation of dark adaptation.

4. For one observer an increase of one photon in the retinal illumination during light adaptation produced an approximately uniform increase in the perception times of 0.05 % (e.g. an increase in pupil diameter from

2.5 to 3.0 mm. during light adaptation would produce an increase in the perception times of 54 %).

5. The correlation of pupil diameter in the dark with perception time for six selected test-patch brightnesses was not significant. This was due to the existence of a significant correlation between pupil diameter in the light and that in the dark. Differences of pupil diameter in the light were eliminated by (a) partial correlation, and (b) correction of the data by the rule stated in the previous paragraph. The corrected data then yielded a series of negative correlation coefficients which increased with the duration of dark adaptation. Thus a comparatively large pupil diameter in the dark tends to decrease perception times, as one would expect.

6. No significant coefficients resulted from the correlation of general colouring or, within the narrow limits examined, of visual acuity with perception time, either with the original, or with the corrected data.

7. Correlation of retinal pigmentation, judged by the difficulty of seeing the choroidal vessels, with perception time, yielded six negative coefficients of which two were significant. There is thus some evidence that heavy pigmentation of the retina favours more rapid dark adaptation.

8. In the second group, there were twenty-six observers of both sexes, aged 17-70 yr. Four highly significant positive coefficients resulted from the correlation of age with perception time for four selected test-patch brightnesses.

Since there is a significant decrease in pupil diameter in the light with age, this slowing of the course of dark adaptation with age cannot be due to pupil variations. Pupil diameter in the dark also decreases with age.

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612.86:595.772 *Drosophila*

## Further studies on pre-imaginal olfactory conditioning in insects

BY W. H. THORPE, M.A., PH.D.

*From the Department of Entomology, Zoological Laboratory, Cambridge*

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In the course of an earlier paper (Thorpe and Jones 1937), it was shown that the ichneumonid parasite *Nemeritis canescens* (Grav.) can be induced to develop a positive olfactory response to the odour of an abnormal host, the wax moth *Meliphora grisella* (F.), by rearing it artificially upon that host. It was shown that the change in the olfactory response brought about by this process was due, in large part, to conditioning taking place in the adult stage immediately after emergence from the pupa. This was confirmed by the fact that *Nemeritis*, reared on the normal host *Ephestia*, can be made positively responsive to the odour of *Meliphora* merely by placing them immediately on emergence in an apparatus through which is pumped a stream of air which has previously passed over a number of living *Meliphora* larvae. But the conditioning, obtained by treatment of the newly emerged adult in this manner, is not usually as strong as that produced by actually rearing the insects on the abnormal host. From this it was concluded that part at least of the conditioning effect produced by rearing on the wax moth larvae must be the result of the influence of the host acting during the pre-imaginal period. Attempts to get conclusive demonstrations of this in *Nemeritis* were, however, ineffective. It was

hoped that it would be possible, having reared the parasite to the pupal stage on the wax moth, to dissect it from its cocoon, which of course may be presumed to be contaminated with the odour of the host. For if pupae, having been thus dissected out and then washed and completely isolated from the odour of the wax moth, still show significant preference for it on emergence, the existence of pre-imaginal conditioning could be considered firmly established. Unfortunately, the high mortality caused when pupae are treated in this way made the experiment impracticable. Many parasites can be removed from their cocoons without suffering harm, but *Nemeritis* appears much more susceptible to injury than most.

The object of the present work was to perform a similar experiment on some insect which does not spin a cocoon and in which the puparia can therefore be washed free of all contaminating odoriferous substances. Because of its convenience as a laboratory insect *Drosophila melanogaster* Mg. (*fasciata* Mg., *ampelophila* Loew.) was chosen for this purpose (a strain being kindly supplied by Dr C. H. Waddington). The two sexes were used indiscriminately since in some preliminary control experiments no significant differences in their behaviour were observed. In a second paper (Thorpe 1938), it was shown that in *Nemeritis* a positive conditioning can be produced, not merely to the odour of a possible host, but to the odour of such biologically abnormal substances as cedar wood oil. It was felt that *Drosophila* also offered a particularly suitable subject for further experiments of this nature in that its rearing on synthetic foodstuff has long been standardized. Odoriferous substances can easily be mixed with the food, thus exposing the larva to their influence over the whole of its life. The *Drosophila* were reared on Pearl's S. 101 medium, modified in certain particulars by Mr J. H. Sang, for whose help in the matter I am much indebted. The exact composition of the medium is as follows:

Potassium sodium tartrate, $\text{NaKC}_4\text{H}_4\text{O}_6$	8.0 g.
Ammonium sulphate, $(\text{NH}_4)_2\text{SO}_4$	2.0
Magnesium sulphate, $\text{MgSO}_4$	0.5
Calcium chloride, $\text{CaCl}_2$	0.25
Tartaric acid, $\text{CHOH}, \text{COOH}_2$	5.0
Monobasic potassium phosphate, $\text{KH}_2\text{PO}_4$	0.65

The medium is made up to 1000 c.c. with distilled water and then autoclaved. Then 80 c.c. of the mixture are taken and 4 g. of agar and 6 g. of household (beet) sugar are added. When these are dissolved the mixture is made up to 100 c.c. with water and 2% of yeast is then added. The mixture is allowed to stand for 4 hr. and is then boiled and put into

bottles. In the preliminary experiments a number of odoriferous substances were tested. The choice is of course limited by the fact that the substance must be, to some extent, water soluble and also non-toxic. This rules out many materials which might otherwise have proved convenient. Those which appeared most promising were artificial musk, vanilla, and essence of peppermint, but of these the last was found to be by far the most satisfactory, and the use of the others was abandoned. It was found that 0.5 % peppermint essence added to the culture medium produced a strong odour without hindering\* the normal development of the larvae. Concentrations higher than 0.5 % tended to be toxic. The technique of the experiments was identical with that described recently (Thorpe 1938), except that an upright olfactometer with a slightly smaller bore was used. Control experiments, which are summarized in Table I, show that normal *Drosophila*,

TABLE I. SUMMARY OF CONTROL EXPERIMENTS. *DROSOPHILA* REARED FROM NORMAL ARTIFICIAL MEDIUM AND TESTED IN OLFACTOMETER. 5 % PEPPERMINT AGAINST "BLANK"

Date 1938	No. in peppermint arm	No. in "blank" arm	Total	No. of exps. in series	No. insects used
19 Sept.	51 = 23.7 %	164	215	3	80
19 Oct.	72 = 24.6 %	221	293	3	120
20 "	42 = 22 %	149	191	4	60
25 "	76 = 33.2 %	153	229	3	90
25 "	145 = 43.7 %	187	332	6	70
25 "	54 = 45.8 %	64	118	2	70
27 "	95 = 47.3 %	106	201	3	70
27 "	77 = 55.4 %	62	139	4	70
28 "	18 = 22.5 %	62	80	2	40
28 "	42 = 25.9 %	120	162	4	65
28 "	43 = 38.0 %	70	113	3	50
9 Nov.	70 = 32.4 %	146	216	4	60
16 "	54 = 33.6 %	107	161	3	60
16 "	36 = 33.0 %	73	109	2	60
21 "	63 = 50 %	63	126	2	75
21 "	61 = 45.0 %	75	136	2	85
21 "	26 = 25.0 %	75	101	2	60
22 "	65 = 45.5 %	78	143	3	50
22 "	52 = 28.4 %	131	183	3	80
24 "	79 = 30.8 %	178	257	5	60
6 Dec.	87 = 36.2 %	153	240	3	100
Total	1308 = 34.9 %	2437	3745	66	1475
	S.E. = $\pm 0.822$ %				

\* The essence of peppermint used consisted of 10 % by volume of "English White" oil of peppermint in 90 % alcohol. The oil itself contained 46 % free menthol and 9 % as ester.

reared on S. 101 without peppermint, are definitely repelled by this odour when they encounter it in the olfactometer. For this purpose, two drops of a 5 % dilution of the essence in absolute alcohol was allowed to evaporate on 2 sq. cm. of no. 1 Whatman filter paper, which was then placed in one of the bait tubes of the instrument, the other arm being left blank. From Table II it will be seen, in a similar series of experiments in which are

TABLE II. *DROSOPHILA* REARED FROM ARTIFICIAL MEDIUM CONTAINING  $\frac{1}{2}$  % PEPPERMINT AND TESTED IN OLFACTOMETER 5 % PEPPERMINT AGAINST "BLANK"

Date 1938	No. in peppermint arm	No. in "blank" arm	Total	No. of expts. in series	No. insects used
A. Fresh from culture					
18 Oct.	115=71.5 %	46	161	3	60
24 "	134=63.8 %	76	210	3	90
24 "	77=65 0 %	42	119	3	40
26 "	174=66.5 %	88	262	4	100
26 "	74=63.8 %	42	116	3	40
28 "	118=69.4 %	52	170	3	70
2 Nov.	97=67.9 %	46	143	5	60
2 "	73=63.5 %	42	115	3	50
4 "	120=57.9 %	87	207	4	60
15 "	117=78.5 %	32	149	3	70
8 Dec.	47=81.0 %	11	58	4	17
Total	1146=67.0 %	564	1710	38	657
s.e.= $\pm 1.213$ %					
B. 1-2 days isolated					
19 Oct.	72=68.0 %	34	106	3	60
20 "	57=62.0 %	35	92	3	40
1 Nov.	67=39.2 %	104	171	5	50
1 "	65=48.1 %	70	135	3	50
Total	261=51.7 %	243	504	14	200
s.e.= $\pm 2.23$ %					
C. 3-4 days isolated					
31 Oct.	96=44.8 %	118	214	5	70
31 "	91=39.3 %	141	232	5	70
3 Nov.	121=47.1 %	136	257	4	80
Total	308=42.9 %	395	703	14	220
s.e.= $\pm 1.89$ %					
D. 6 days isolated					
3 Nov.	118=35.7 %	213	331	6	100
s.e.= $\pm 2.75$ %					

used insects reared on a medium containing peppermint, that the result is entirely different. If the insects are fresh from culture, instead of being repelled by the odour they are now attracted to it to the extent of 66.45 %. This positive conditioning does not persist, however, if the insects are isolated, and at the end of 6 days the figure is brought down very nearly to that shown by the controls.

TABLE III. *DROSOPHILA* REARED FROM ARTIFICIAL MEDIUM CONTAINING  $\frac{1}{2}$  % PEPPERMINT. PUPAE WASHED IN DISTILLED WATER AND ISOLATED UNTIL EMERGENCE. TESTED IN OLFACTOMETER. 5 % PEPPERMINT AGAINST "BLANK"

Date 1938	No. in peppermint arm	No. in "blank" arm	Total	No. of exps. in series	No. insects used
21 Oct.	40 = 40.0 %	59	99	3	30
22 "	76 = 44.2 %	96	172	4	40
7 Nov.	113 = 60.1 %	75	188	4	60
8 "	91 = 49.0 %	95	186	4	60
10 "	91 = 49.5 %	94	185	5	50
12 "	72 = 50.3 %	56	128	3	60
14 "	66 = 49.3 %	68	134	4	50
16 "	91 = 71.6 %	36	127	3	60
17 "	99 = 55.6 %	79	178	5	50
18 "	72 = 54.5 %	65	137	4	45
19 "	100 = 53.5 %	87	187	5	50
Total	911 = 53.0 %	809	1720	44	555
	S.E. = $\pm 1.213$ %				

TABLE IV. *DROSOPHILA* REARED FROM ARTIFICIAL MEDIUM CONTAINING  $\frac{1}{2}$  % PEPPERMINT. FULLY FED LARVAE WASHED IN DISTILLED WATER AND ISOLATED UNTIL EMERGENCE OF THE ADULT. TESTED IN OLFACTOMETER. 5 % PEPPERMINT AGAINST "BLANK"

Date 1938	No. in peppermint arm	No. in "blank" arm	Total	No. of exps. in series	No. insects used
1 Dec.	37 = 22.3 %	129	166	4	50
2 "	141 = 60.8 %	91	232	4	70
3 "	119 = 73.0 %	44	163	3	65
3 "	136 = 62.4 %	82	218	4	65
5 "	115 = 57.2 %	86	201	4	60
Total	548 = 55.9 %	432	980	19	310
	S.E. = $\pm 1.6$ %				

If, now, insects are removed from the peppermint cultures immediately after pupation and are washed three times in distilled water and isolated, we arrive at the results obtained in Table III. It will be seen that, although



the difference between the percentage attracted to the peppermint and that going to the "blank" arm is barely significant, it is still positive and greatly different from the controls. Similarly, Table IV shows that washing fully-fed larvae, and isolating them before pupation, still does not eliminate the conditioning effect. This is important; for while it is difficult

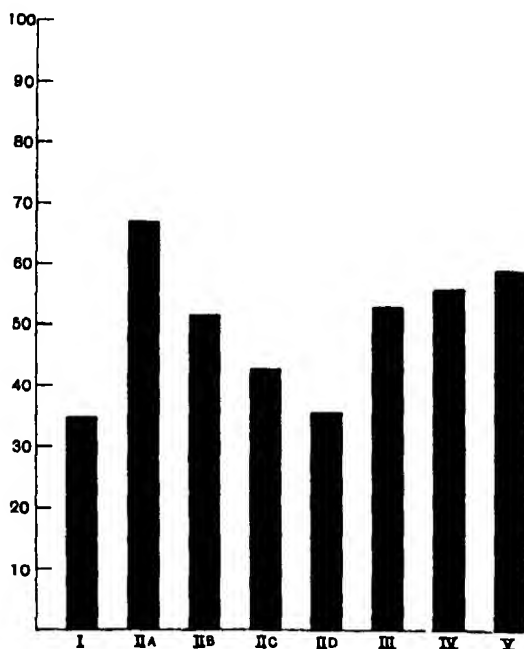


FIG. 1. Diagram to summarize results of Tables I-V. Height of columns indicates percentage of *Drosophila* attracted to odour of peppermint in olfactometer. I. Controls reared from normal medium and tested fresh from culture. II. Insects reared from medium containing 0.5% peppermint. A. Tested fresh from culture. B. Tested after 1-2 days isolation. C. Tested after 3-4 days isolation. D. Tested after 6 days isolation. III. Insects reared from medium containing 0.5% peppermint. Puparia washed in distilled water and isolated. Tested upon emergence. IV. Same as III except that insects washed as fully fed larvae. V. Insects reared on normal medium but adults exposed to vapour of peppermint for 2-5 days immediately upon emergence.

to wash the puparia completely clean, larvae taken direct from the medium can easily be freed from all traces of it. From these results, which, as the figures show, are highly significant, we can conclude that pre-imaginal conditioning is actually in operation. The rate of extinction of the conditioning effect is summarized in fig. 1. It will be seen that it is closely similar to that obtained with *Nemeritis*.

It was shown (Thorpe 1938) that *Nemeritis* can be induced to give positive responses to a new odour, even if it only meets it as a constituent of a generally favourable environment. That is to say, in order to produce that effect it is not necessary for the new odour to be specifically associated with any particular act such as feeding or oviposition. Moreover, it was pointed out that such a mechanism, which is not a simple reflex association but rather the acceptance of a new factor as part of a favourable environment *as a whole*, provides a satisfactory basis for explaining the remarkable behaviour of ants in mixed colonies. It is of interest to see that the same phenomena can be shown in *Drosophila* and that if the adult insects are exposed on emergence to a stream of air containing peppermint vapour they will lose their repugnance for it and develop a positive conditioned response. Table V gives clear evidence of this.

TABLE V. *DROSOPHILA* REARED ON NORMAL ARTIFICIAL MEDIUM AND EXPOSED TO VAPOUR OF PEPPERMINT IN "CONDITIONER" FOR 2-5 DAYS IMMEDIATELY ON EMERGENCE. TESTED IN OLFACTOMETER. 5% PEPPERMINT AGAINST "BLANK"

Date 1938	No. in peppermint arm	No. in "blank" arm	Total	No. of exps. in series	No. insects used
11 Nov.	183 = 59.0 %	128	311	4	100
14 "	82 = 59.0 %	57	139	5	40
18 "	73 = 59.4 %	50	123	4	40
Total	338 = 59.0 % S.E. = $\pm 2.09$ %	235	573	13	180

#### DISCUSSION

The same objection that applies to *Nemeritis* as a subject for research of the type here described might perhaps be thought to apply also to *Drosophila*; for the pupa of the latter is enclosed within its puparium just as that of the former is within its cocoon. Undoubtedly it would have been preferable to have used an insect with a naked pupa had one suitable in other respects been available. But deficiency in the essential qualities of rapid breeding and ease of culture rule out many otherwise promising species.

Since urates and uric acid have sometimes been found in the ecdysial fluid of insects it has been thought that the so-called moulting glands, the openings of which are distributed over the body surface, might be excretory in function. It has, however, been shown by Shimizu (1931) and confirmed by Esperon (1937) that in the silkworm this is not the case. In this insect

the contents of the Malpighian tubes pass via the rectum into the space between the old and new cuticle, at the time of moulting, and become spread out in a layer in the ecdysial fluid. If the same process were taking place in *Drosophila*, the experiments described in this paper might be unreliable as evidence of larval conditioning; for the peppermint from the faeces might come into contact with the whole cuticle of the pupa and thus bring about a conditioning shortly before the emergence of the adult from the puparium. That escape of faecal matter into the ecdysial fluid is not the explanation of the deposit of calcium carbonate on the puparium of diptera has been shown by the work of Keilin (1921) on *Acidia*. Eastham (1925) has also shown that the contents of the Malpighian tubes of *Calliphora erythrocephala* and *Drosophila funebris* never reach the puparium at all but are passed to the exterior in the usual manner. I have confirmed these conclusions in *D. melanogaster* by the following simple experiment.

*Drosophila* larvae are fed on the usual medium to which sufficient carmine has been added to give a bright red colour. The puparia are washed free of all medium and then dissected. No deposit of carmine granules is found spread over the inner surface of the puparium although, of course, the faeces are brightly coloured. The rectum is emptied prior to pupation and, since the insect has now left the medium, this faecal material tends to spread out over the *outside* of the terminal segment of the puparium and colour it red. A very small quantity may remain inside the cast rectal lining in between the pupa and puparium. A few granules of this carmine may sometimes escape but do not spread farther than the immediate neighbourhood of the puparial anal scar, certainly never beyond the terminal segment. Similarly, a very little carmine may occasionally be left in the cast lining of the oesophagus, but this does not appear to spread at all.

In the previous experiments with *Nemeritis* no definite evidence of transference of the changed responses from one generation to the next was obtained, and indeed the shortness of duration of the effect in the adult life would in any case render this unlikely. The question of the influence of such treatment on the second and subsequent generations of *Drosophila* has not actually been tested. But it seems worth considering whether the mechanism of pre-inaginal conditioning, such as has here been demonstrated, might not explain some of the results of Sladden and Hower on the transference of the induced food habit of the stick insect *Carausius morosus*. If we assume that the new food plant, i.e. ivy, contains a chemical substance of particularly persistent and penetrating qualities, it might be possible to imagine enough of this substance being contained in the egg to

produce positive conditioning in the newly emerged nymph. This might be particularly the case in those insects in which the first food consists of the empty egg shell.

The diametrically opposed conclusion reached by McIndoo (1935), in his work with the Colorado beetle *Leptinotarsa decemlineata*, appears to be unique and does not at present seem capable of explanation. McIndoo gives some evidence for the existence of a conditioned *repugnance* for egg laying on species of plants on which individuals had been reared. But more investigation seems to be required.

#### SUMMARY

1. Previous work on positive pre-imaginal olfactory conditioning of the ichneumonid parasite *Nemeritis canescens*, by exposure to the odour of unusual chemical substances, has been repeated, using *Drosophila melanogaster*. After tests with a number of substances, 0.5 % of peppermint essence (containing 4.6 % free menthol) was found to be satisfactory when mixed with the synthetic food medium.

2. All the main conclusions established by the work on *Nemeritis* have been confirmed even more strikingly with *Drosophila*.

3. Although normally repelled by the odour of peppermint, flies which have been reared on a medium containing 0.5 % peppermint essence are markedly attracted by the odour of this substance in an olfactometer.

4. Conditioning brought about in this way gradually becomes extinct if the insects are isolated and has practically disappeared in about 6 days.

5. Washing the fully-fed larvae, or newly-formed puparia, free from the medium does not eliminate the conditioning effect although it reduces it to some extent. It is, therefore, concluded that a change in the responses of the adult can be brought about by an influence operating only during the larval life.

6. It has been shown that with *Drosophila*, as with *Nemeritis*, exposure of the adult to the odour of the substance immediately after emergence from the pupa will bring about a positive conditioning even though the odour is not specifically associated with any particular favourable quality of the environment.

In conclusion I wish to express my gratitude to Dr A. D. Imms for his helpful interest in the investigation and for kindly seeing the paper through the press during my absence abroad. Thanks are once again due to the

laboratory assistant, Mr F. Bloy, without whose constant skilled help in care of stocks and working of apparatus the work would not have been completed.

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# The mechanical efficiency of frog's muscle

By A. V. HILL, SEC.R.S.

(From the Department of Physiology, University College, London)

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The factors determining the maximum work and the mechanical efficiency of muscle were discussed by Hartree and Hill in 1928. Work was measured with the Levin-Wyman ergometer (Levin and Wyman 1927), the muscle being allowed to shorten at constant speed, adjustable as required, and recording a tension-length curve on a smoked surface. The whole of the work which the muscle can do in a single shortening at the given speed is thus obtained, as in no other method. The mechanical efficiency, therefore, should be the highest possible.

The efficiencies given by Hartree and Hill, however, were considerably too low, probably owing to an error in the heat measurements: their thermopile was not "protected" against temperature differences along the muscle (cf. Hill 1937, 1938). The part of the muscle off the thermopile being warmer than the part on it would cause too large a temperature deflexion when it shortened on to the thermopile, so making the observed heat too great. The highest efficiency they found for the initial process in frog's muscle was about 26 %, or for the whole cycle, including recovery, about 13 %. This is low compared with measured efficiencies in man, where values as high as 22 % seem to be authentic. It was desirable, therefore, to repeat the measurements on frog's muscle with a protected thermopile.

Recent work (Hill 1938) on the energetics of muscular shortening has made it possible to give a theoretical treatment of the present problem, and from known data to calculate approximately the most efficient speed, the optimum load, and the absolute value of the efficiency. We will consider the initial process only, and assume that the muscle shortens at a constant speed  $v$ , through a distance  $x$ , that the relation between speed of shortening and force exerted is given by the characteristic equation

$$(P + a)(v + b) = (P_0 + a)b, \quad (1)$$

and that the heat given out is independent of the work done and is made up of two terms, one, the heat of maintenance, being proportional to the



duration  $t$  of the shortening and equal to  $kt$ , the other being the heat of shortening  $ax$ . We will examine these assumptions in detail later.

The work done in shortening is

$$W = Px.$$

The heat given out is  $H = ax + kt$ .

The efficiency is  $E = \frac{W}{H+W} = \frac{Px}{(P+a)x + kt}$ .

If the speed of shortening is high the time is short and the term  $kt$  in the denominator is small. The force exerted, however, is also small at a high speed, so the numerator is small too and the efficiency low. If, on the other hand, the speed is low  $P$  is large, but  $t$  is large too, and again the efficiency is low. At some intermediate speed the efficiency is a maximum, and the calculated relation between efficiency and speed is shown in fig. 1. This is obviously similar in general to the relation found by Dickinson (1929) and by Lupton (1923) between efficiency and speed in human movement. There is also an optimum load for an isotonic contraction, and the calculated relation between efficiency and load is shown in fig. 2.

The calculation is made as follows. Dividing the numerator and denominator by  $t$  and writing  $v$  for  $x/t$  we obtain

$$E = \frac{Pv}{(P+a)v + k}.$$

Using the characteristic equation (1) we may eliminate  $v$  and obtain

$$E = 1 - \frac{(1+a/P_0)a/P_0}{(1+a/P_0+K/P_0)(P/P_0+a/P_0)} - \frac{(1+K/P_0)K/P_0}{(1+a/P_0+K/P_0)(1+K/P_0-P/P_0)}, \quad (2)$$

where  $K = k/b$ . This gives the efficiency in terms of load (as a fraction of the full isometric tension  $P_0$ ). Or we may eliminate  $P$  and obtain

$$E = \frac{1 - \frac{a}{P_0}v}{1 + \frac{a}{P_0} + \frac{K}{P_0} + \frac{K}{P_0}v}. \quad (3)$$

This gives the efficiency in terms of speed (as a fraction of the characteristic velocity  $b$ ). The load for maximum efficiency is given by

$$\frac{P/P_0 + a/P_0}{1 + K/P_0 - P/P_0} = \sqrt{\frac{(1+a/P_0)a/P_0}{(1+K/P_0)K/P_0}}, \quad (4)$$

and the speed for maximum efficiency by

$$\frac{b}{v} = \frac{a}{P_0} \left\{ 1 + \sqrt{\frac{(1+a/P_0)(1+K/P_0)}{aK/P_0^2}} \right\}. \quad (5)$$

The maximum efficiency itself is

$$E_{\max} = 1 - \left[ \frac{\sqrt{\{(1+a/P_0)a/P_0\}} + \sqrt{\{(1+K/P_0)K/P_0\}}}{1+a/P_0+K/P_0} \right]^2. \quad (6)$$

We see that the efficiency depends on the relative load  $P/P_0$ , or the relative speed  $v/b$ , and on the two constants  $a/P_0$  and  $K/P_0$ .

Now these two constants are approximately known for frog's muscle at  $0^\circ \text{C}$ .  $a/P_0$  is about 0.25 and  $K = k/b$ ,  $b$  being about one-third of the muscle length per second (Hill 1938). It is necessary, therefore, to know  $k/P_0$ . In a number of experiments performed during 1938 the heat liberated at  $0^\circ \text{C}$  in isometric contractions of various durations of stimulus had the mean values given in Table I. The heat reckoned per second of stimulus is greater for shorter durations than for long ones. A contraction, however, outlasts the stimulus, and a muscle at  $0^\circ \text{C}$  may go on shortening and doing work for a time of the order of 0.3 sec. after the stimulus is over. In the third row, therefore, the heat is reckoned per second of contraction, obtained by adding 0.3 sec. to the duration of the stimulus. The numbers so obtained are nearly constant, and we may take a mean value of 165. This is reckoned per gram of muscle. Let a muscle be  $l$  cm. long and  $A$  sq. cm. in cross-section. Taking the density of muscle as 1.05, the heat production per second of contraction is  $k = 165 \times 1.05 \times l \times A$  g. cm. Now  $b$  is  $l/3$  per sec. so that  $K = k/b = 520 A$  g. wt. The average value of  $P_0$  in frog's muscle at  $0^\circ \text{C}$  is about 1600 g. wt per sq. cm. of cross-section ( $a/P_0 = 0.25$ ,  $a = 400$ ; Hill 1938), so that in a muscle of cross-section  $A$  sq. cm. it is 1600  $A$  g. wt. Hence  $K/P_0 = 520/1600 = 0.325$ .

TABLE I

Stimulus: seconds	0.6	0.8	1.0	1.2	1.4
Heat: g.cm. per g. per sec. of stimulus	247	230	215	202	190
Heat: g.cm. per g. per sec. of contraction	165	167	165	162	157

This value is confirmed by Feng's (1931) results. A recalculation of the quantities in his paper gives, for the slope of the relation between  $H/P_0 l$

and  $t$  (the duration of stimulus), a temperature coefficient of 3.0 per 10° C and an absolute value at 0° C of 0.107. Now  $K/P_0 = k/bP_0 = 3k/lP_0$  (taking  $b = l/3$  per sec.) and  $k$  is  $H$  per sec. during the steady slope. Thus  $K/P_0 = 3 \times 0.107 = 0.321$ , which is close to the value just calculated.

From these values,  $a/P_0 = 0.25$ ,  $K/P_0 = 0.325$ , the efficiencies given in Table II have been calculated. It will be noted that the calculated efficiency is independent of the duration of the stimulus, and depends only on the speed of shortening, or alternatively on the load. The optimum load in an isotonic contraction is 0.475 of the full isometric tension. The optimum speed with the Levin-Wyman ergometer is 0.725 of the characteristic velocity  $b$ , or in a muscle of 3 cm. length 7.25 mm./sec. The maximum efficiency is 40.5 %. Within limits, which will be referred to below, these predictions are rather accurately verified by experiment. We see, moreover, that the efficiency remains high for some way on either side of the maximum, being above 39 % from  $v/b = 0.5$  to 1.0, or from  $P/P_0 = 0.58$  to 0.37. A speed, or a load, anywhere near the optimum should give very nearly the maximum efficiency.

TABLE II. CALCULATED EFFICIENCY OF FROG'S SARTORIUS MUSCLE AT 0° C FOR VARIOUS SPEEDS OF SHORTENING, OR FOR VARIOUS LOADS

$v/b$	0	0.05	0.1	0.2	0.3	0.4	0.5
$P/P_0$	1.0	0.94	0.89	0.79	0.71	0.64	0.58
$E, \%$	0	12.2	20.2	29.6	34.7	37.7	39.3
$v/b$	0.6	0.7	0.8	0.9	1.0	1.2	1.4
$P/P_0$	0.53	0.49	0.44	0.41	0.37	0.32	0.27
$E, \%$	40.1	40.4	40.4	40.0	39.5	37.9	36.0
$v/b$	1.6	1.8	2.0	2.5	3.0	3.5	4.0
$P/P_0$	0.23	0.20	0.17	0.11	0.06	0.03	0.0
$E, \%$	33.7	31.3	28.8	21.9	14.8	7.5	0

Maximum efficiency = 40.5 %. Optimum speed:  $v/b = 0.725$ . Optimum load:  $P/P_0 = 0.475$ .

The effect of temperature on the calculated efficiency depends on its effect on  $a/P_0$  and  $K/P_0$ . According to Hill (1938, p. 174),  $a/P_0$  is unaffected by temperature. According to Feng (1931),  $k/P_0$  increases about 3 times for 10° C. According to Hill (1938, p. 174),  $b$  increases about 2.05 times for 10° C. Thus  $K/P_0 = k/bP_0$  should increase somewhat with temperature, which would have the effect of making the maximum efficiency rather lower at a higher temperature. We cannot, however, be sure of this since the effect of temperature on  $b$  is not yet very accurately known.

The calculated efficiency is plotted against speed of shortening in fig. 1, and against isotonic load in fig. 2. These can be compared with the experimental results given below.

In order to get a value for the maximum efficiency of 44 %, corresponding to the 22 % for the complete process (including recovery) in man, it would be necessary (assuming  $a/P_0 = 0.25$  as in frog's muscle) to take a value of  $K/P_0$  of about 0.25. For  $K/P_0 = 0.25$  the maximum efficiency is 44.4 %, the optimum load is  $P/P_0 = 0.5$  and the optimum speed  $v/b = 2/3$ . In man, therefore, we might expect to find the optimum load for a maximal effort to be just half the maximum load that can be lifted and the optimum speed to be one-sixth of the maximum speed ( $4b$ ) under zero load.

The argument given above has been intentionally over-simplified in order to provide a manageable result. We have assumed:

(1) that the whole muscle is governed by the characteristic equation relating force and speed. actually only the contractile part is so governed, the elastic part introduces a small but not negligible complication;

(2) that the characteristic equation applies for any amount of shortening, and that  $P_0$ , the full isometric tension, is the same at all lengths: actually  $P_0$  usually becomes slightly greater at first as the length diminishes, and then less again, reaching zero at about 40 % shortening;

(3) that the isometric heat rate ( $k$ ) is the same at different lengths: actually it is greater after a certain amount of shortening, but considerably less after still further shortening (Hill 1938, p. 172; and unpublished experiments by Brown and Katz).

In the following experiments the effect of (1) has been largely avoided in the experiments with the Levin-Wyman ergometer by a slightly delayed release, while in isotonic contractions it has been approximately allowed for. As regards (2), the variation of  $P_0$  with length would be exactly balanced if  $a$  and  $K$  varied in the same way, i.e. if  $a/P_0$  and  $K/P_0$  in equations (2) to (6) were independent of length. The fact that, over rather a wide range, the efficiency proves to be independent of the duration of stimulus (and therefore of the amount of shortening) suggests that this is approximately true. The equations assume that the muscle can go on shortening indefinitely. Actually  $P_0$  becomes zero after about 40 % shortening and  $K$  does not. consequently the efficiency is lowered if the stimulus is uselessly continued after this amount of shortening has occurred. No interest, therefore, attaches to longer stimuli with higher speeds. As regards (3), the way in which  $k$  depends upon length is roughly parallel to the way in which  $P_0$  does. This would tend to make  $K/P_0$  independent of length ( $K = k/b$ ) as was suggested above. How  $b$  varies with length has not been investigated.

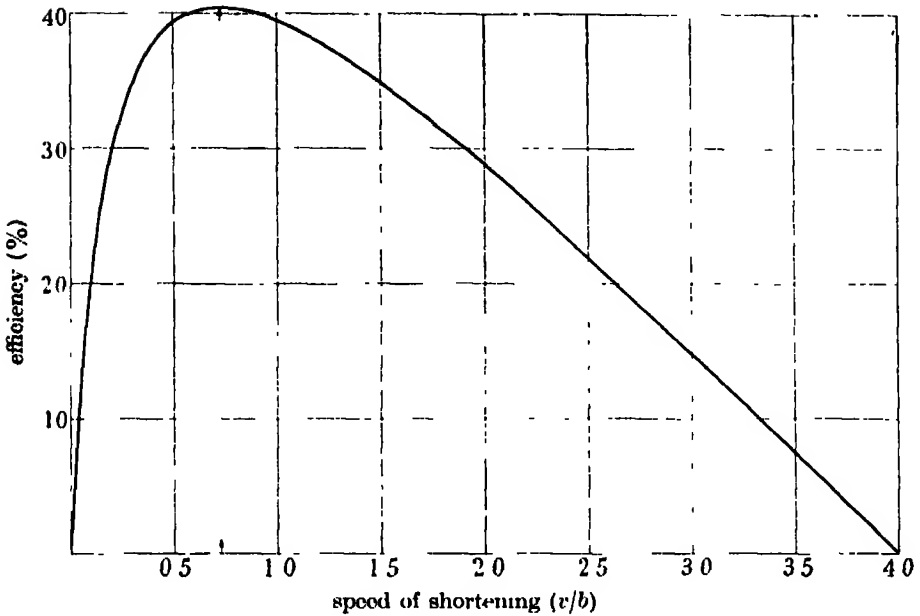


FIG. 1. Calculated relation between mechanical efficiency (initial process) and speed of shortening in frog's muscle at 0° C. Optimum speed shown by arrow.

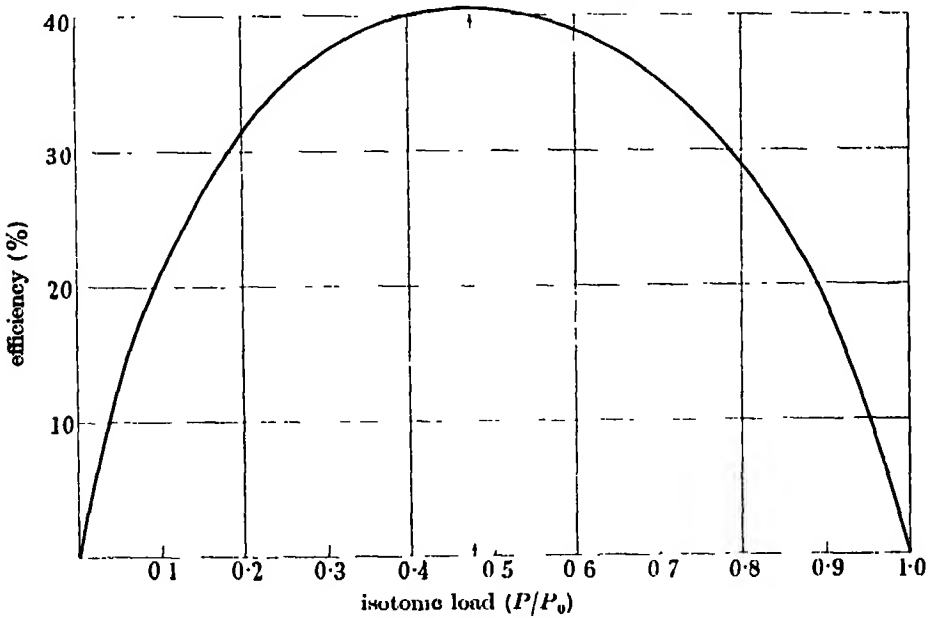


FIG. 2. Calculated relation between mechanical efficiency (initial process) and load in isotonic contraction of frog's muscle at 0° C. Optimum load shown by arrow.

Not enough is yet known about these various disturbing factors to make it useful to try to include them in the equations. The real question is why a theory which takes no account of them fits the experimental facts so closely. Some such balancing out of the different factors must provide the answer. This is not an uncommon experience.

### METHOD

*A. Experiments with the Levin-Wyman ergometer.* The ergometer, described by Levin and Wyman (1927) and used by Hartree and Hill (1928) in their experiments on mechanical efficiency, is released by a magnet at any desired moment and then moves at a constant speed determined by the setting of a needle valve. It carries a tension lever on which the muscle pulls, and a tension-length curve is written on a smoked glass plate. The speed was measured by a stop-watch graduated in 0.02 sec. over the measured distance between the release and a screw stop.

A set of curves is given in fig. 3 for the 0.5 sec. tetanus of a 33 mm. muscle shortening at 0° C at the following speeds: 1.5, 2.6, 4.6, 7.2, 9.9, 13.4, 15.5, 21.5 and 26.4 mm./sec. In every case the muscle contracted isometrically for 0.1 sec., developed a tension of 35–40 g., and was then released. With the lowest speed of shortening the tension rose to nearly its full isometric value, but relaxation occurred before much work was done, and the shortening was only 2.3 mm. With higher and higher speeds the tension developed became less and less but the amount of shortening more and more: at 21.5 mm./sec. 13.7 mm. shortening occurred, or 42% of the length of the muscle.

The work done is given approximately by the area of the tension length curve. If the coordinates were rectilinear and the lever gave strictly proportional deflections the relation would be exact. Actually the axes are arcs of circles, and the deflection is not strictly proportional to tension. A grid, therefore, was constructed as shown in the figure, the lines of constant tension being made by hanging known weights on the lever and allowing the ergometer to run down, the lines of constant shortening by letting the ergometer down to the screw stop adjusted to various distances and pulling on the lever. The sides of each "square" in the grid represent 5 g. tension and 1.417 mm. shortening respectively, and the corresponding work is 0.709 g.cm. The total work for any of the curves is obtained by adding up the squares or fractions of a square under it, and multiplying by 0.709. The grid was made on smoked glass, projected by a lantern and

drawn on paper: the curves were made on similar smoked glass, projected on the grid and measured.

It was necessary to give the muscle a small constant load in order to stretch it out initially to a constant length and bring it back when it

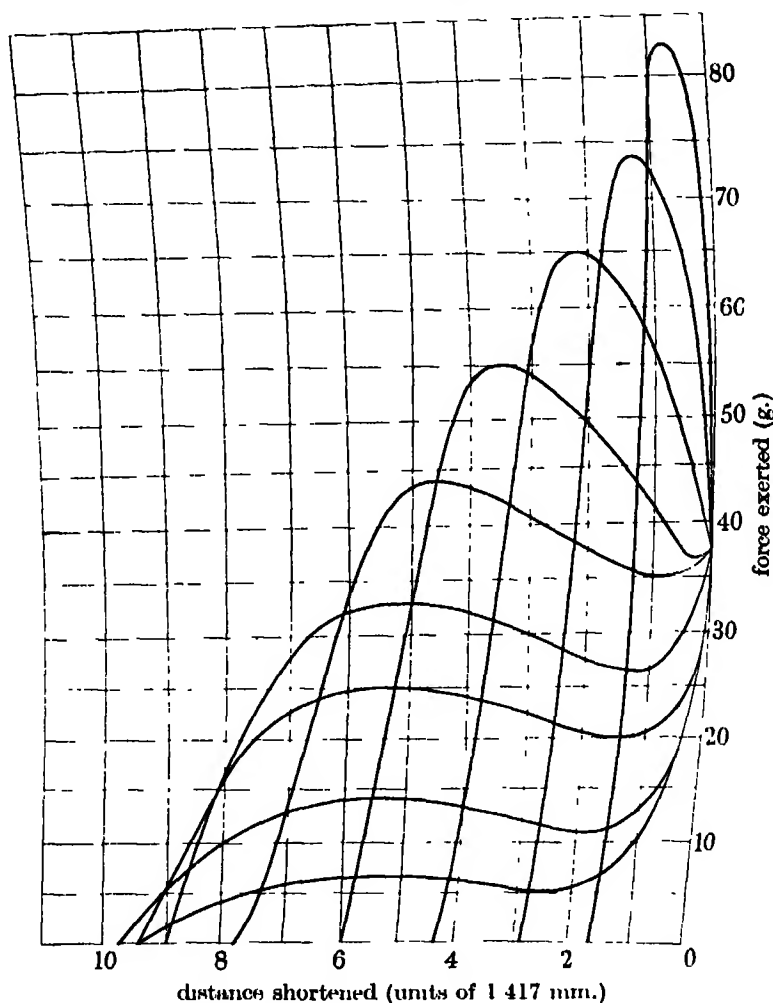


FIG. 3. Curves obtained with Levin-Wyman ergometer for muscle shortening at various speeds; projected on measuring grid. See text.

relaxed. In the experiment shown in fig. 3 the load was 3.3 g. The work done on this load was obtained by multiplying it by the total distance it was lifted, as shown by the point where the curve cut the horizontal axis. This work was added to that obtained from the area of the curve.

The thermopiles were of the "protected" type: P 5 (constantan-manganin) was about  $45\mu$  thick and equivalent to  $34\mu$  of muscle, it gave  $1628\mu\text{V}/1^\circ\text{C}$ , had a resistance of 68 ohms, and allowed a "protected" shortening of 29% of the muscle length (see Hill 1938, p. 141); P 6 (palladium gold-iron) was about  $40\mu$  thick and equivalent to  $31\mu$  of muscle, it gave  $1176\mu\text{V}/1^\circ\text{C}$ , had a resistance of 15.5 ohms, and allowed a "protected" shortening of 38%. The thermopile was connected to a moving coil galvanometer of 1.28 sec. period and readings were made on a scale every 3 sec. from the beginning of the stimulus, for 24 sec. The logarithm of the deflexion was plotted against time and the line so obtained extrapolated back to the middle of the stimulus. In this way heat loss and inequalities of heat production were allowed for (Hill 1939). The readings were turned into g.cm. for comparison with the work.

The stimulus was provided by maximal alternating condenser discharges (10-15 each way per sec.) of about minimum energy ( $RC$  = about 1 msec.). The heat produced by the stimulus was usually negligible but could be allowed for if necessary.

The muscle contracted isometrically at first for about 0.1 sec. and was then released. During this interval it shortened against the elasticity of the chain and lever and of its own tendons, and developed one-third to one-half of its full isometric tension. After release it shortened further against the ergometer moving at constant speed. The delay in release should not be too short, for otherwise the muscle may complete too much of its shortening before its tension is sufficiently developed: it should not be too long, for otherwise too large a part of the time available for shortening is wasted. The best delay was found by trial (cf. Hartree and Hill 1928, p. 241).

In calculating the total energy the work determined from the area of the tension length curve was added to the observed heat. The work done against the small constant load was not added, since this was converted into heat during relaxation. The efficiency was calculated by dividing the total work by the total energy.

The speed has been expressed, either in mm./sec. or in terms of  $v/b$ , where  $b$  is taken (at  $0^\circ\text{C}$ ) as one-third of the length of the muscle per sec., so that  $v/b = 3v/l$ .

The efficiency has no significance when the stimulus is continued after the muscle attains its minimum length; for heat is then produced to no useful end. The efficiency, therefore, has been recorded at higher speeds only for shorter stimuli.

*B. Isotonic contractions.* The arrangement used was that previously described (Hill 1938), the "after" load being held up by a magnet released



just before relaxation set in. In this way the work done against the "after" load was not turned into heat during relaxation: that done against the (small) initial load, however, did appear as heat. The work was measured in the usual way from the height of contraction and the total load.

The magnet was released by a third key on the revolving contact breaker: the first and second keys determined the duration of stimulus, the third was opened at a suitable moment (found by trial) after the end of the stimulus, to allow maximum shortening to occur. An arm held back by the magnet sprang rapidly forward on release and held the lever carrying the "after" load against a support, so that the muscle relaxed under the initial load only.

It might appear to be simpler to allow the muscle to relax under the load, so adding the work automatically to the heat and obtaining the total energy from the galvanometer deflexion. It usually happens, however, that relaxation occurs at slightly different moments in different parts of the muscle, the work is transformed into heat very irregularly (cf. Hill 1938, p. 169), and anomalous deflexions result. This trouble is avoided if the load is held up and the work is obtained separately.

With the greater "after" loads an appreciable amount of work is done by the muscle in stretching its tendons and the chains and levers attached to it: this energy appears as heat in relaxation, but is not measured directly as work. It has been allowed for approximately as follows. The muscle is stimulated isometrically and develops a force  $P_0$  g. wt. It is then suddenly released. It shortens at first very rapidly through a distance  $h_0$  cm. and then more slowly. The first is due to passive release of tendons, etc., the second is due to active contraction. If we assume that the elastic stretch  $h$  is proportional to the load  $P$ , then  $h = h_0 P/P_0$  and the mechanical energy stored is  $Ph/2 = P^2 h_0/2P_0$ . This was added to the work. The allowance is not very exact, but the quantity is not large and no serious error occurs.

The heat was measured as described above, but the method of allowing for heat loss had not been realized at the time of these experiments which were earlier than those with the Levin-Wyman ergometer. The heats, therefore, are rather too small and the efficiencies slightly (1 or 2%) too high. Since the apparatus for the isotonic contractions had been dismantled, and the results with the ergometer were much more exact, it was not considered worth while to repeat the earlier experiments.

## RESULTS

In fig. 4 are four typical curves showing the relation experimentally observed between efficiency and speed. They include extreme examples,

chosen to show the range of variation. The results are not plotted for speeds so high that the muscle shortened completely before the contraction ended, and the curves cannot properly be continued to the right.

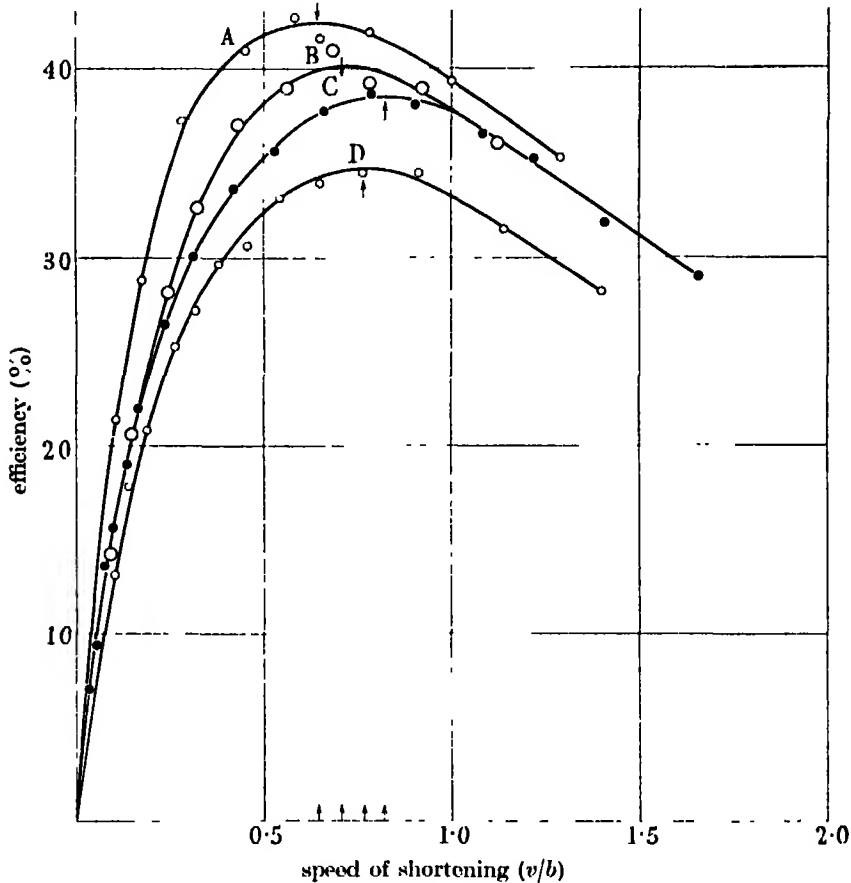


FIG. 4. Experimental relation between mechanical efficiency (initial process) and speed of shortening in frog's muscle at  $0^{\circ}\text{C}$ . Four experiments; extreme examples, high and low, to show variation. Optimum speeds shown by arrows. A, 0.6 sec. stimulus, 0.12 sec. release, thermopile P 6. B, 0.8 sec. stimulus, 0.13 sec. release, thermopile P 6. C, 0.5 sec. stimulus, 0.10 sec. release, thermopile P 5. D, 0.6 sec. stimulus, 0.12 sec. release, thermopile P 5.

It is clear that the experimental relation is very similar to the calculated one shown in fig. 1; not only in general shape but (a) in the absolute value of the maximum and (b) in the speed at which the maximum occurs. Curve C is for the experiment of which some of the work curves are given in fig. 3.

The results of ten such experiments are given in Table III. In each case a series of measurements was made for a constant duration of stimulus but with variable speed of shortening, the results were plotted and the maximum efficiency and optimum speed read off as from the curves of fig. 4. The mean value of the maximum efficiency was 39.4 % and of the optimum speed  $v/b = 0.735$ , very close to the predicted values of 40.5 % and  $v/b = 0.725$  respectively.  $b$  has been taken as  $l/3$  per sec., so that  $v_{opt} = 0.245l/\text{sec.}$  Full shortening is usually about  $0.37l$  which occurs at this speed in 1.5 sec., so that tetani much longer than 1.2 sec. will give artificially low values. Very short tetani did not yield sufficiently consistent results, so that the mean has been taken only for tetani of duration 0.4–1.2 sec. Actually for speeds somewhat less than the optimum quite high values of the efficiency can still be obtained with longer stimuli, for example, in Table III, 36.9 % for 1.4 sec., 39.3 % for 1.5 sec. and 37.7 % for 2.4 sec.

TABLE III. MAXIMUM EFFICIENCY AND OPTIMUM SPEED WITH THE LEVIN-WYMAN ERGOMETER

Tetanus: sec.	0.5	0.5	0.8	1.1	0.2	0.4	0.7	1.0
Release: sec.	0.1	0.11	0.13	0.15	0.09	0.1	0.13	0.16
Max. efficiency: %	38.6	40.0	40.2	39.4	37.0	38.0	38.0	38.0
Optimum speed: $v/b$	0.82	0.78	0.65	-	-	0.87	0.78	0.66
Tetanus: sec.	0.6	1.0	1.5	0.6	1.0	0.6	1.0	1.4
Release: sec.	0.12	0.16	0.20	0.12	0.20	0.15	0.20	0.25
Max. efficiency: %	42.4	43.0	39.3	34.7	42.0	37.3	38.2	36.9
Optimum speed: $v/b$	0.66	0.61	—	0.80	0.75	0.76	0.69	—
Tetanus: sec.	0.6	1.2	2.4	1.2	1.2			
Release: sec.	0.15	0.20	0.3	0.2	0.2			
Max. efficiency: %	39.9	39.9	37.7	40.0	39.8			
Optimum speed: $v/b$	0.78	0.66	—	0.69	0.78			

Mean maximum efficiency for all tetani of 0.4 to 1.2 sec. = 39.4 %. Mean optimum speed for the same tetani,  $v/b = 0.735$ .

Apart from the complicating factors discussed above (p. 438) theory predicts that the efficiency should be independent of the duration of stimulus. At a given speed more work would be done with a longer stimulus and correspondingly more heat would be liberated. The chief of these factors is the condition that a muscle cannot shorten more than a certain amount, and that the efficiency is necessarily lowered when the contraction is continued after maximum shortening has occurred. Avoiding such contractions, the results in Table IV show that the efficiency is, in fact, highly independent of the duration of the stimulus. The speeds in

Exp. 1 and 2 were chosen to be about the optimum. In (1) the work varied from 20.1 g.cm. for the 0.2 sec. to 52.6 g.cm. for the 1.0 sec. tetanus; yet the efficiency was constant. In (2) the work varied from 22.3 to 51.0 g.cm. In Exp. 3 *a* the speed was far below the optimum, so that 3.0 sec. was a possible duration: in Exp. 3 *b* the speed was rather above the optimum, 1.2 sec. was already rather too long and the efficiency had begun to fall. It is very striking how constant, for a given speed, the efficiency is when the duration of stimulus (and therefore the amount of shortening) is varied. Turning to equation (3) above it is difficult to see any simple reason for this other than that  $a/P_0$  and  $K/P_0$  should be, approximately at least, independent of length. This conclusion should be tested directly.

TABLE IV. EFFECT OF DURATION OF STIMULUS ON EFFICIENCY

(1) Constant speed, $v/b = 0.745$									
Tetanus: sec.	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Efficiency: %	36.2	37.0	36.6	36.9	37.7	37.5	37.9	38.4	37.4
(2) Constant speed, $v/b = 0.75$									
Tetanus: sec.	0.3	0.5	0.7	0.9	1.1	1.3			
Efficiency: %	37.8	38.5	38.2	38.3	37.9	37.8			
(3) (a) Constant speed, $v/b = 0.3$									
Tetanus: sec.	1.0	2.0	3.0						
Efficiency: %	31.3	32.8	32.7						
(3) (b) Constant speed, $v/b = 0.93$									
Tetanus: sec.	0.4	0.6	0.8	1.0	1.2				
Efficiency: %	40.6	41.5	41.2	41.3	40.2				

With isotonic contractions the relation between efficiency and load is of the general form predicted in fig. 2. The optimum load is just in the right place (predicted  $P/P_0 = 0.475$ , mean observed 0.46) but the absolute value of the efficiency is much lower (see Table V). Moreover, the efficiency is by no means independent of the duration of stimulus, having (for a given load) a clear—if rather blunt—maximum, depending on the load. The higher the load the greater the optimum duration of the stimulus.

The discrepancy is probably due to the fact that the isometric tension  $P_0$  varies considerably with length, so that to obtain the highest efficiency the load (as a given fraction of  $P_0$ ) ought to rise somewhat at first as shortening proceeds, and then fall towards zero as the muscle tends to its minimum length. With a constant load the muscle lifts it at first at a constant speed, then comes to a stage at which it lifts it more slowly and finally cannot lift it at all. With a greater constant load the muscle

shortens more slowly and so takes longer to reach a length at which  $P_0$  is seriously diminished: the optimum duration is therefore greater. With the Levin-Wyman ergometer the muscle is never prevented from shortening, since the force opposing it is not constant but simply what the muscle itself can exert at the given speed. It can continue to shorten and do work even while its tension is falling during relaxation: with a constant load it cannot, so that the work is less.

TABLE V. MAXIMUM EFFICIENCY AND OPTIMUM SPEED IN ISOTONIC CONTRACTIONS

Exp. no.	...	1			2	3	4		
Tetanus: sec.		0.3	0.5	0.8	0.9	0.6	0.15		
Max. efficiency: %		28	34	32	33	37	32		
Optimum load: $P/P_0$		0.42	0.45	0.50	0.46	0.47	0.44		
Exp. no.	...	5				6	7	8	
Tetanus: sec.		0.15	0.2	0.35	0.5	0.8	1.0	0.8	1.1
Max. efficiency: %		34	34	35	32	32	37	33	35
Optimum load: $P/P_0$		0.44	0.43	0.53	0.57	0.38	0.47	0.44	0.49

Mean value of optimum  $P/P_0 = 0.46$ . Mean value of maximum efficiency = 33 %.

We may consider the matter from the point of view of equation (2) above. If, as seems likely, from the results with the Levin-Wyman ergometer,  $a/P_0$  and  $K/P_0$  are approximately independent of length, there is only one quantity in equation (2) which depends on length, viz.  $P/P_0$ , which for a constant load  $P$  increases considerably as the muscle shortens and  $P_0$  diminishes. If, therefore,  $P/P_0$  is about an optimum at the start it rises beyond the optimum, in the end considerably beyond, as shortening proceeds. Consequently the efficiency diminishes in successive elements of the shortening and the net efficiency of the whole process is less than the theoretical maximum. If the load starts considerably below the optimum it may indeed reach the optimum later in the shortening, but the efficiency is now low in the earlier stages, and the net efficiency of the whole process is again reduced. It is, in fact, impossible to choose a constant load which remains an optimum at all lengths, and the net efficiency of the whole isotonic contraction is bound to be less than the theoretical value. With the ergometer it is the velocity which is constant, not the load, and apparently about the same optimum speed exists at all lengths during the shortening. Consequently a true maximum efficiency can be obtained.

No great interest, therefore, is attached to the experimental relation between efficiency and load in an isotonic contraction: the condition of a

$P_0$  varying with length is too complex to treat theoretically. It is very fortunate that with the ergometer and a constant speed of shortening the complexity which might have arisen from a variation of  $P_0$  with length is apparently balanced by approximately parallel variations of  $K$  and  $\alpha$ .

The fact that in isotonic contractions the optimum load has about the theoretical value may be attributed to two experimental facts: (a) that the optimum speed of shortening has about the theoretical value, and (b) that the characteristic equation (1) is rather accurately obeyed during the earlier stages of shortening. The optimum load can be calculated from the optimum speed by equation (1).

### DISCUSSION

In a previous paper (Hill 1939) it was shown that during a steady state of activity due to contractions at regular intervals, with somewhere near maximum work performed, the total energy is rather exactly equal to twice the initial energy. The mechanical efficiency, therefore, of the whole muscular process, including recovery, is simply obtained by dividing the efficiency observed for the initial stage by 2. Thus the maximum efficiency of the whole process of contraction in frog's muscle at  $0^\circ\text{C}$  is about 20 %.

Many estimates of the mechanical efficiency have been made in man, particularly with a bicycle ergometer. Since a single contraction cannot be studied, but only a long series of contractions, the absolute value depends largely on the "base line" assumed in the calculation for those normal metabolic processes which are unchanged in muscular work. The maximum value obtained in Dickinson's (1929) investigation, by what is probably the most reliable method, was about 21.5 %, and this occurred at an optimum speed corresponding to a time for one leg movement of 0.9 sec. The absolute value is only slightly higher than in frog's muscle. In human movements, however, a fraction of the energy is used in circulatory and respiratory effort, and in the maintenance of the posture of the body, over and above the energy used for these purposes at rest. It is impossible to allow for this separately. It is probable, therefore, that the efficiency of the human muscle itself is slightly higher than that of the frog's, but not so much higher as earlier determinations on frog's muscle suggested. The two efficiencies are clearly of the same order of size and probably determined by similar factors.

The maximum efficiency depends (see equation (6) above) on  $\alpha/P_0$  and  $K/P_0$ . If these were less the efficiency would be greater; for example, if  $\alpha/P_0$  and  $K/P_0$  were both 0.3 the maximum initial efficiency would be 39 %, P

if they were both 0.2 it would be 51 %, if they were both 0.1 it would be 69 %. There is no direct evidence at present as to the values of  $a/P_0$  and  $K/P_0$  in human muscle: to give the observed efficiency, however, they should be slightly less than in frog's muscle at 0° C.

In frog's muscle at 0° C the speed for maximum efficiency is given by  $v/b = 0.725$ . From equation (5) the optimum speed can be calculated for any values of  $a/P_0$  and  $K/P_0$ , and if these vary together the optimum speed  $v/b$  alters only little as they alter. for example, if  $a/P_0 = K/P_0 = 0.1$ , the optimum speed is  $v/b = 0.83$ : for  $a/P_0 = K/P_0 = 0.2$  the value is 0.71: for  $a/P_0 = K/P_0 = 0.3$  the value is 0.67. It is probable, therefore, that in man the optimum speed is  $v/b =$  about 0.7. Now the observed optimum time for one movement in Dickinson's experiments was 0.9 sec. The amount of shortening is not known: let us for illustration take it to be 20 % of the length of the muscle = 0.2*l*. The optimum speed  $v$  is then  $0.2l/0.9 = 0.22l$  per sec. If this is equal to  $0.7b$  we find  $b = 0.31l$  per sec., which is about the same as in frog's muscle at 0° C. The amount of shortening cannot well have been more than 0.3*l* or less than 0.1*l*, so that  $b$  must lie within the limits 0.47*l* and 0.16*l* per sec. In frog's muscle at human body temperature the value of  $b$  (with a temperature coefficient of 2.05) would be much greater than this.

The value of  $b$  in man can be roughly estimated from independent data. The time required to complete a maximum unloaded shortening of human arm muscles is about 0.25 sec. (Lupton 1922). If we suppose that the amount of shortening is one-quarter of the muscle's length then the maximum speed (unloaded) is  $l$  per sec. Now from the characteristic equation (1) above the maximum speed is  $P_0b/a$  and if  $P_0/a = 4$  as in frog's muscle this is  $4b$ . Hence  $4b = l/\text{sec.}$ , or  $b = 0.25l$  per sec. This is in the middle of the range we calculated above, 0.16–0.47*l* per sec.

These calculations about human muscle are given as illustrations only, and to indicate the probable order of quantities. It seems likely that  $a/P_0$  and  $K/P_0$  are between 0.2 and 0.3, and that  $b$  is of the order of one-quarter of the muscle length per sec. A direct investigation of these quantities should be possible: for example  $a$  and  $b$  might be determined by finding experimentally the relation between load and speed of shortening and applying the characteristic equation (1). Knowing  $b$ ,  $K = k/b$  could be found by measuring  $k$ , e.g. with a thermocouple inserted in a muscle during a maximal isometric contraction. The direct determination of these "dynamic constants" on human muscle would be of the greatest interest.

One peculiar coincidence, if it be a coincidence, has been realized in this work, viz. that  $a/P_0$  and  $K/P_0$  are very nearly equal: they might have been

completely different. In frog's muscle at  $0^{\circ}\text{C}$  we have taken  $a/P_0 = 0.25$  and  $K/P_0 = 0.325$ . If we took both as 0.287 (their mean value) we should not be outside the range of possibility in the experimental measurements, and the calculated values of the maximum efficiency and of the optimum speed would be unchanged. If in fact they were equal, since  $K = k/b$  we should have  $a = k/b$ , or  $k = ab$ . Thus the heat required per sec. to maintain an isometric contraction would be equal to the "heat of shortening" at velocity  $b$ . There is no apparent reason why  $k$  should be even of the same order of quantities as  $ab$ . That it is in fact nearly if not exactly equal to  $ab$  is very suggestive.

The approximate equality may be a chance occurrence. It may, however, depend upon some fundamental property of muscle. During an isometric contraction in outward appearance a muscle is still. The energy required to maintain the contraction at constant length is about equal to the extra heat for shortening at velocity  $b$ , a velocity which is as characteristic in muscle as the velocity of sound is in air. In a maintained contraction the ordered molecules are relaxing and being restored all the time, the process being accompanied by a continual liberation of heat. This heat is the same, or nearly the same, as the extra heat accompanying the redistribution and reorientation of molecules in shortening at velocity  $b$ .

The apparent relation between  $k$  and  $ab$  should be explored in other muscles, and in frog's muscle at other temperatures. If an approximate equality is not found elsewhere it must be regarded as a coincidence in frog's muscle at  $0^{\circ}\text{C}$ . If it persists it may yield an important clue as to the mechanism of the contractile process.

#### SUMMARY

1. A theoretical discussion is given of the mechanical efficiency of muscle, based upon the dynamic constants  $a$  and  $b$  of a previous paper, the isometric tension  $P_0$ , and the heat of maintenance  $k$ . Equations are given relating the efficiency to the speed in a contraction at constant speed, and the efficiency to the load in an isotonic contraction. The constants are known for frog's muscle at  $0^{\circ}\text{C}$  and absolute values of the efficiency are calculated.

2. Experiments are described on frog's muscle at  $0^{\circ}\text{C}$ , using a "protected" thermopile and a Levin-Wyman ergometer, from which it appears that the relation between efficiency and speed is of the predicted form, and that the maximum efficiency and the optimum speed are very close to the predicted values.



3. At a given speed the observed efficiency is independent of the duration of stimulus, and therefore of the amount of shortening. The cause of this is discussed.

4. In isotonic contractions the optimum load agrees closely with that predicted, but the maximum efficiency is less. The divergence is explained.

5. The observed maximum efficiency (40 %) of the initial process corresponds to an efficiency of 20 % for the whole muscular cycle including recovery. This is not much less than the maximum efficiency in man.

6. The maximum efficiency depends only on  $a/P_0$  and  $K/P_0$  where  $K = k/b$ . It appears that  $a/P_0$  and  $K/P_0$  are approximately independent of length. In frog's muscle at  $0^\circ$  C  $k$  is approximately equal to  $ab$ ; in other words, the heat required to maintain a contraction is about equal to the extra heat associated with shortening at velocity  $b$ .

7. Approximate estimates are given of the dynamic constants of human muscle, which seem to be similar to those of frog's muscle at  $0^\circ$  C.

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# The development of the Weberian ossicles and anterior vertebrae in the goldfish

By J. M. WATSON, A.R.C.S.

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## INTRODUCTION

The study of the development of the anterior part of the vertebral column in the goldfish, *Carassius auratus* (L.), presents an interesting problem on account of the modification associated with the presence of the Weberian ossicles. Although much descriptive and anatomical work has been done on the Weberian ossicles, the early stages of their development have been described only by Nusbaum (1881) in the carp and by Matveiev (1929) in the rudd, who are by no means wholly in agreement with regard to the method of origin. Some difference of opinion also exists with regard to the existence of separate intercalary elements in the anterior part of the vertebral column of teleosts.

In this investigation of the development of the anterior vertebrae and Weberian ossicles of the goldfish it has been my endeavour to elucidate these points.

The young stages of the goldfish were reared in open-air ponds at Beam Brook, Newdigate. Experiment showed that length was a better criterion of degree of development than age, so larvae of various sizes were obtained by using fine-meshed nets in the breeding ponds. The study of the development was made entirely by means of sections. The young fish were embedded by the benzol-wax method and the sections were stained in Delafield's haematoxylin. All sections were examined by the preparation of camera-lucida drawings, graphic reconstructions and photographs.

## THE DEVELOPMENT OF THE ANTERIOR VERTEBRAE

Immediately upon hatching the young fish is 3–4 mm. in length, and the vertebral column is represented only by the notochord and its sheaths. By the 8 mm. stage the basidorsals and basiventrals of the first five vertebrae

are present, but the independent mesenchymatous rudiments which contribute to the formation of the Weberian ossicles do not appear until the 10 mm. stage.

*The first vertebra*

The anterior vertebrae are separated from the musculature by the saccus paravertebralis. In the adult no neural arch corresponds to this vertebra, and in the 11 mm. stage the spinal cord is surrounded by a peculiar ring of cartilage which is continuous anteriorly with the cranial cartilage (fig. 1).

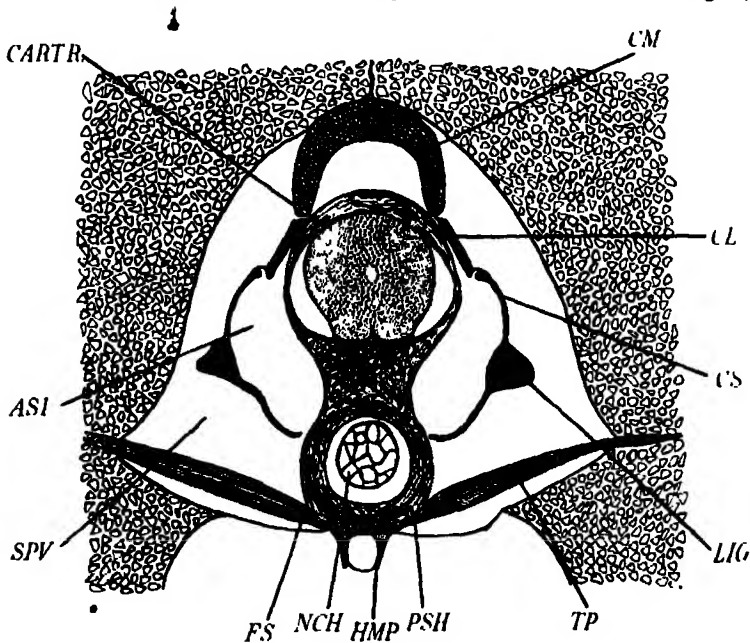


FIG. 1. Diagrammatic transverse section in the region of the first vertebra of a 12 mm. goldfish to show the rudiments of the claustrum and scaphium. (The parapophyses are not shown since they do not lie in the plane of the section.)

The thickened base of this ring of cartilage lies between the notochord and the spinal cord and extends up on either side of the latter so that in cross-section the cavity appears to be semicircular. It arises independently of the rest of the first vertebra and can be traced forwards into the skull where it becomes flattened out and forms the roof of the *cavum sinus imparis*. It may represent a non-bony extension of the exoccipitals. Just in front of the first vertebra it narrows and the atria of each side pass below it and unite to form the *sinus imparis*. In the adult the base of the ring becomes converted into fibrocartilage in the vertebral region and forms a kind of pad between the spinal cord and the centrum. In the skull region

it becomes ossified into a thin plate of bone, roofing over the *cavum sinus imparis*.

The claustrum rudiment and the scaphium rudiment are present in this region, and that part of the basidorsal of the first vertebra which would normally give rise to the neural arch becomes part of the scaphium rudiment by fusion with the independent mesenchymatous anlage of the concha stapedis (figs. 1, 2, 5).

The lower arch or basiventral of a vertebra may give rise either to a parapophysis, which is lateral or ventrolateral in position, or to a haemapophysis, which is ventral in position, or to both; and either or both of these structures

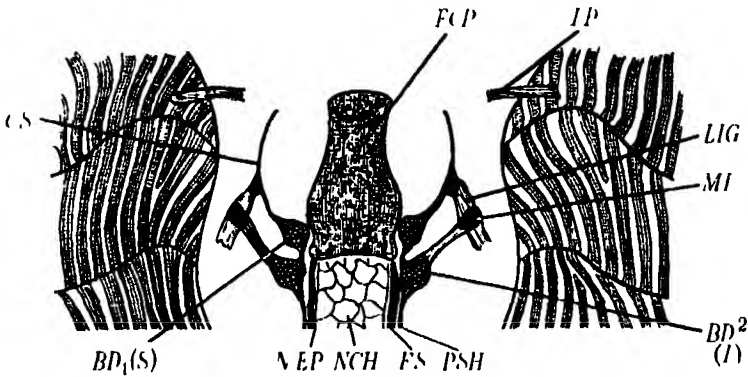


FIG. 2 Diagrammatic frontal section of the region of the first two vertebrae in a 12 mm. goldfish to show the fibrocartilage pad, the rudiments of the scaphium and the intercalarium, and the tips of the "transverse processes" of the first vertebra.

may be cartilaginous or mesenchymatous. Both occur in the typical trunk vertebrae of the goldfish and both are present in the first vertebra. The parapophyses are massive lateral blocks of cartilage while the haemapophyses are small mesenchymatous processes of the ventral surface of the centrum (11 mm. stage).

Lying toward the anterior margin of the centrum and proceeding outwards from the cartilaginous parapophyses are the so-called "transverse processes" which are mesenchymatous in the 11 mm. stage but later undergo ossification. In the adult they are firmly fused to the centrum proximally while distally they penetrate the myotomes. The homologies of these processes have been much disputed. Their structure indicates that they are ribs, and their position, attached to the middle of the centrum and penetrating the muscles, points to homology with the dorsal or upper ribs of *Polypterus* (Budgett 1902) and of *Esox* and other teleosts (Goodrich 1930) (figs. 1, 2).

*Second and third vertebrae*

In the 11 mm. stage the developing centra of the second and third vertebrae are distinct and separate, with corresponding sets of cartilaginous arcualia. The arcualia of the second vertebra contribute to the intercalarium rudiments while the arcualia of the third vertebra contribute to the tripus rudiments. Above these vertebrae lies a large mass of cartilage which, in the adult stage, when the centra of the second and third vertebrae have fused, becomes ossified to form the neural spine and arches of the

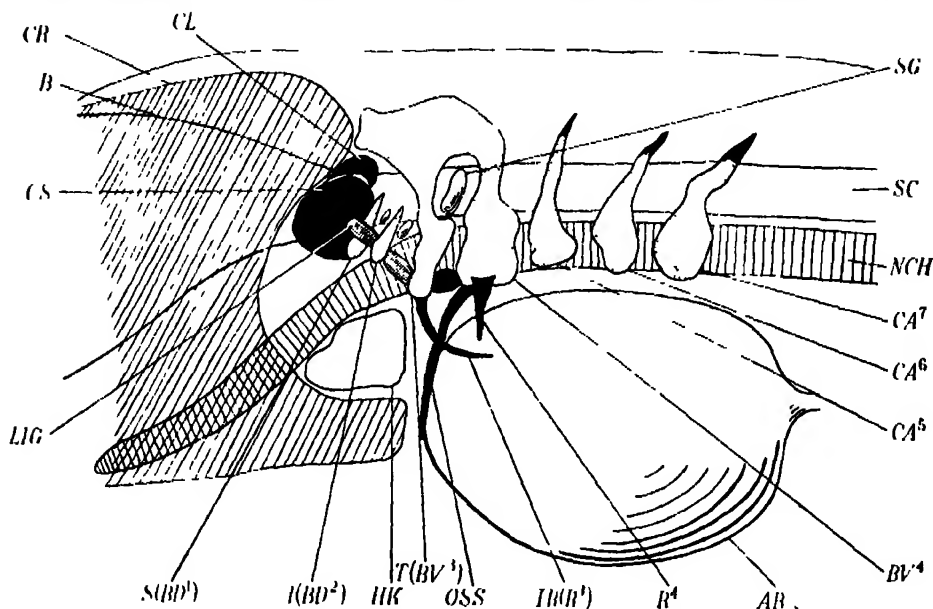


FIG. 3. Reconstruction from sagittal sections of the anterior vertebral region in a 12 mm. goldfish, illustrating the form of the rudiments of the Weberian ossicles and their relationships to the other structures in this region.

"compound" vertebra. The basidorsal of the fourth vertebra is fused with this mass, as is also part of the basidorsal of the second vertebra. The absence of interspinous bones corresponding to the first three vertebrae, whether as rudiments or in the adult stage, indicates that these also are probably fused into the mass early in ontogeny. This structure therefore probably represents the fused neural spines and basidorsals of the second, third and fourth vertebrae together with the first three interspinous bones and possibly the neural spine of the first vertebra (fig. 3).

In the adult stage the centra of the second and third vertebrae become fused so as to form one large amphicoelous centrum, above which the

cartilage mass develops into the compound neural arch and an enormous neural spine anteriorly, to which is closely applied, posteriorly, a much smaller neural spine belonging to the fourth vertebra. From the anterior margin of the compound vertebra, i.e. the true second vertebra, arises a pair of processes similar to and homologous with the "transverse processes" of the first vertebra.

#### *Fourth vertebra*

The fourth vertebra is less modified than its predecessors and in the adult stage is typical, except that the ribs are shorter and that a pair of triangular processes arise from the ventral surface. In the 12 mm. stage it is clear that the ribs, which have been sometimes referred to as "transverse processes", are true pleural ribs, since proximally they are formed from the basi-ventrals, while distally they are composed of strands of mesenchyme similar in form and position to those which give rise to the ribs further back; moreover, they pass below the musculature.

The rudiments of the ossa suspensoria are visible in the 11 mm. stage as narrow strands of mesenchyme sloping forwards and downwards from their point of origin, the perichordal sheath on the ventral side of the developing fourth centrum (fig. 4). These structures resemble the haemapophyses of the first vertebra in form and origin and are undoubtedly homologous with them.

#### *Summary table*

The following table indicates the fate of the various rudiments of the anterior vertebrae.

Vertebra	Basidorsal	Parapophysis	Haemapophysis
1	Scaphium	Base of "transverse process"	Present
2	Cartilage mass and intercalarium	Base of "transverse process"	Present
3	Cartilage mass	Tripus	Absent
4	Cartilage mass	Head of rib	Ossa suspensoria
5	Typical neural arch and spine	Head of rib	Present

*Note.* The cartilage mass referred to in the table above gives rise, as has been pointed out, to the neural arches and spines of the second, third and fourth vertebrae. The participation of the basidorsal of the second vertebra was not detected in the young stages, but was inferred by reason of the fact that the second vertebra does possess a neural arch, unlike the first vertebra, and since that neural arch does originate from the cartilage mass.

### THE DEVELOPMENT OF THE WEBERIAN OSSICLES

In the 11 mm. stage the rudiments of the Weberian ossicles are present but fusion of parts has not yet begun, so that interpretation is facilitated.

#### *Claustrum*

The claustrum rudiment lies between the first and second vertebrae, and slightly overlaps both. In the anteroposterior direction it is co-extensive with the scaphium. It occupies the same position and has much the same form as in the adult. It arises from an accumulation of mesenchyme cells that become directly ossified, and forms the upper part of the inner wall of the atrium sinus imparis, the lower part being formed by a thin sheet of mesenchyme which extends downwards to join the block of cartilage lying below the spinal cord. Ventrolaterally the claustrum rudiment is attached to the scaphium rudiment by a thin sheet of mesenchyme while dorsally it is joined by mesenchyme to the vault of cartilage which lies over the spinal cord (figs. 1, 3, 5).

#### *Scaphium*

Just below the claustrum rudiment, a circular accumulation of loose mesenchyme cells appears in the 10 mm. stage. It is entirely independent of the vertebral structures in this region at first. It lies in a parasagittal plane and later becomes ossified to form the concha stapedis. The cartilaginous basidorsal of the first vertebra, lying just behind the mesenchyme rudiment of the concha stapedis, becomes fused with this rudiment in the 11 mm. stage. The basidorsal then projects dorsally and ventrally beyond the margin of the circular mesenchymatous mass and later gives rise dorsally to the ascending process and ventrally to the articulating process. As development proceeds the whole scaphium rudiment becomes ossified and forms a unified structure showing no trace of its dual origin (figs. 1-3).

To the outer surface of the concha stapedis rudiment becomes attached the interossicular ligament which passes backwards, downwards and outwards to become attached posteriorly to the basiventral of the third vertebra.

#### *Intercalarium*

The intercalarium first appears in the 12 mm. stage as a direct ossification in the interossicular ligament quite close to the concha stapedis rudiment. It projects beyond the ligament on the inner side, has an oblong form and lies at an angle of about 45° to the sagittal plane with the anterior end outwardly directed. This ossification becomes the manubrium incudis. Opposite to it lies the cartilaginous basidorsal of the second vertebra, with

which it fuses later (15 mm. stage), the whole intercalarium becoming ossified to form a unified structure revealing no trace of its dual origin. The basidorsal of the second vertebra gives rise to the articulating and ascending processes of the intercalarium (figs. 3, 5, 6).

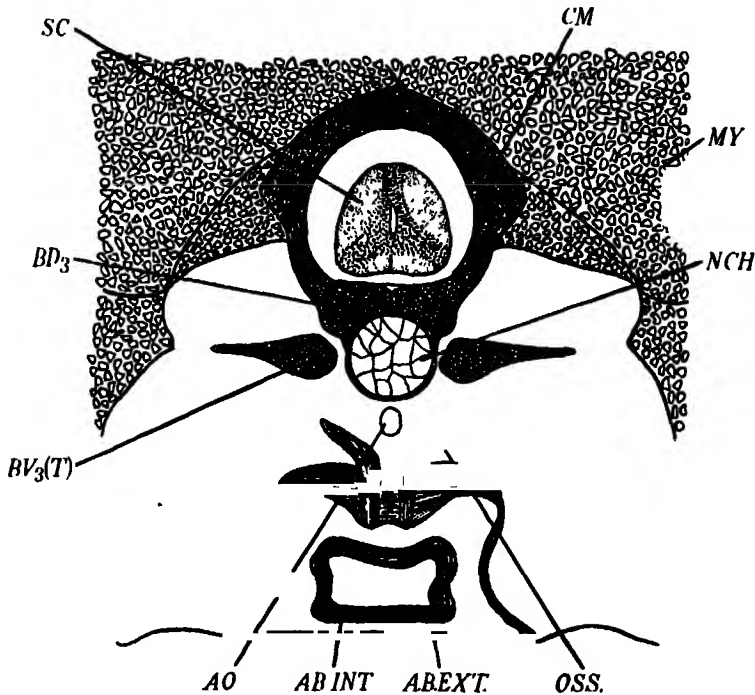


FIG. 4. Diagrammatic transverse section in the region of the third vertebra to show the rudiments of the ossa suspensoria, the tripus and the neural arch and spino (12 mm. stage).

In two families, the Cobitidae and the Siluridae, the intercalarium does not articulate with the vertebral column, but retains its independence as a nodule of bone embedded in the interossicular ligament. The basidorsal of the second vertebra therefore does not participate in the formation of the intercalarium in this case. Thus the adult condition in these families corresponds to the embryonic condition in the goldfish and therefore must be considered primitive.

#### *Tripus*

The basiventral of the third vertebra is a prominent mass of cartilage which projects into the saccus paravertebralis (fig. 4), and is attached anteriorly to the interossicular ligament. Its distal end is formed of mesenchyme which becomes ossified later (15 mm. stage) and curves downwards



and inwards to become attached to the outer coat of the anterior wall of the air-bladder. This is the principal rudiment of the tripus, the cartilaginous basiventral giving rise to the anterior process and the articulating process

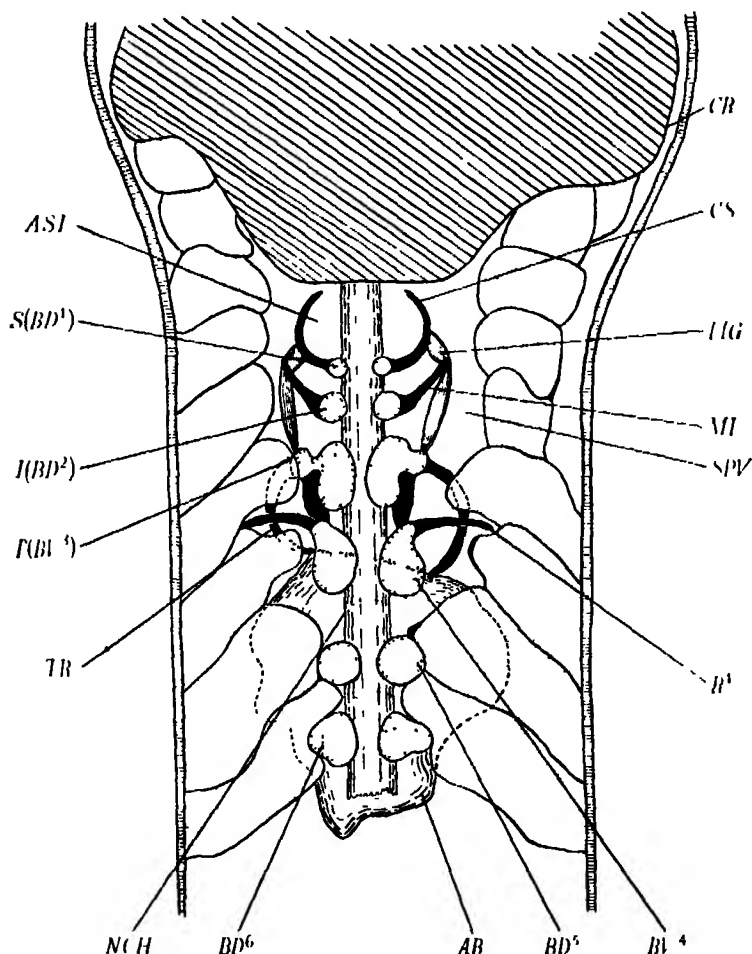


FIG. 5. Reconstruction from frontal sections of the anterior vertebral region in a 12 mm. goldfish, illustrating the method of formation of the Weberian ossicles and the relationships of their rudiments to the air-bladder and the vertebral column.

while the distal mesenchymatous rudiment gives rise to the transformator process. In the 15 mm. stage the transformator process is added to by an ossification arising in the outer coat of the anterior chamber of the air-bladder, while an ossification in the interossicular ligament similar to that which gives rise earlier to the manubrium incudis contributes the tip of the anterior process.

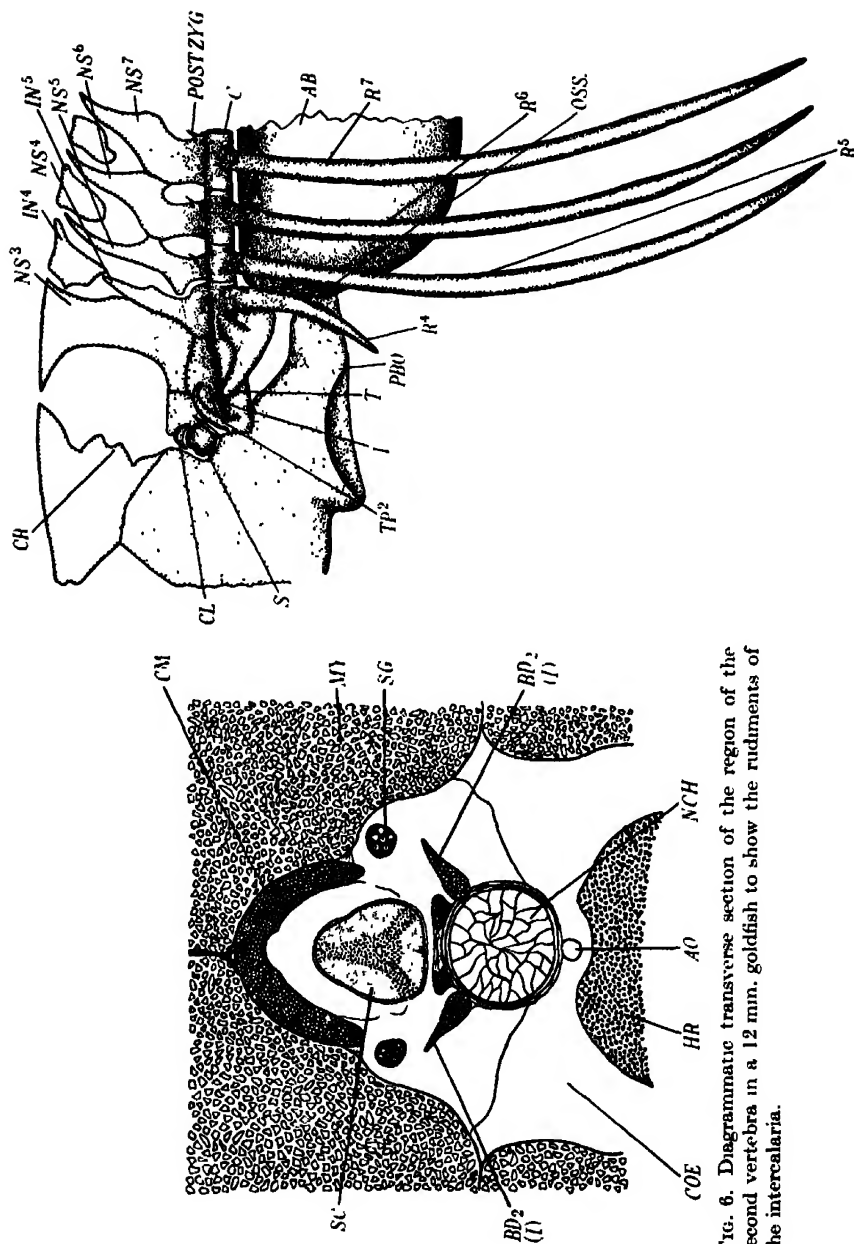


FIG. 6. Diagrammatic transverse section of the region of the second vertebra in a 12 mm. goldfish to show the rudiments of the intercalaria.

FIG. 7. Side view of the back of the skull and the anterior vertebrae in an adult goldfish to show the peculiar spatial relationships of the Weberian ossicles and their connexion with the air-bladder.

Between the basiventrals of the third and fourth vertebrae (11 mm. stage) lies a mass of mesenchyme which is attached to the third basiventral and leads towards the fourth basiventral. This mass later becomes ossified and forms the mass of bone which lies between the proximal end of the transformator process and the articulating process in the fully formed tripus. In the 25 mm. stage, when ossification is complete, it has a fibrous appearance and forms a strut increasing the rigidity of the whole ossicle, holding apart the articulating process and the transformator process which might otherwise become pressed against one another, and converting the tripus into a massive and rigid rather than a thin and elastic structure (figs. 3, 5).

Fusion of the various skeletal elements forming the Weberian ossicles does not become complete until the 20 mm. stage. The following table indicates the nature of the various rudiments that go to make up the Weberian ossicles:

Ossicle	Part	Anlage
Clastrum	—	Crescentic accumulation of mesenchyme in wall of atrium sinus imparis
Scaphium	Concha stapedis Ascending and articulating processes	Circular disc of mesenchyme Basidorsal of the first vertebra
Intercalarium	Manubrium incudis Ascending and articulating processes	Ossification in interossicular ligament Basidorsal of the second vertebra
Tripus	Anterior process	Basiventral of the third vertebra plus ossification in interossicular ligament
	Articulating process	Basiventral of the third vertebra
	Main body	Mass of mesenchyme between third and fourth basiventrals
	Transformator process	Rib rudiment of the third vertebra plus ossification in wall of air-bladder

## DISCUSSION

In discussing the question of the homologies of the various components of the vertebral column much emphasis has been laid by earlier investigators on the tissue of which the structure is composed, whether cartilage or mesenchyme. Matveiev (1929) and Schauinsland (1903, 1906), however, have shown clearly that similar skeletal structures may be composed either of cartilage or of mesenchyme in different regions of the same animal or in

different animals. Thus in the pike (*Esox lucius*) the basidorsals are cartilaginous while the basiventrals are mesenchymatous. Further, in the Clupeidae the arcualia are cartilaginous whereas in the Gadidae they are mesenchymatous. This point is of importance in discussing the homologies of the anterior vertebrae and the components of the Weberian ossicles in the goldfish.

Several authors, notably Matveiev (1929) and Ramanujam (1929), have claimed that dorsal intercalaries occur in the anterior part of the vertebral column in Teleostei. Faruqi (1935), however, has shown that Ramanujam's claim to have found dorsal intercalaries in the first two vertebrae of the herring (*Clupea harengus*) is based on misinterpretation. Matveiev's claim is more serious. He considers the dorsal arch in Cyprinidae to be double and indicates in his diagrams of the young stages of the rudd (*Scardinius erythrophthalmus*) a very marked division into basidorsal and dorsal intercalary. He maintains that in the first three vertebrae there are isolated dorsal intercalaries, while in the posterior vertebrae the dorsal intercalary has become fused with the basidorsal so that only one dorsal arch appears. He interprets this phenomenon in the following way: the double condition of the dorsal arch in fish is a primitive condition which has been lost in the majority of Teleostei; in the Cyprinidae there is secondary recapitulation of the double condition of the arches in the anterior vertebrae because of the presence of the Weberian apparatus, the development of which delays the fusion of the dorsal intercalaries and the basidorsals, which normally takes place very early in ontogeny.

In the goldfish there is no trace, in any stage, of such division in the dorsal arches of the anterior vertebrae. Careful examination of the early stages when the arches are developing from the perichordal sheath failed to reveal more than one pair of dorsal accumulations of mesenchyme per vertebra. Some degree of recapitulation certainly occurs in connexion with the anterior vertebrae, since the basiventrals are cartilaginous in this region, although mesenchymatous further back as in the pike, but no separate dorsal intercalaries occur.

Since it is improbable that the teleostean vertebral column is primitively monospondylous it seems possible that the posterior zygapophysis may represent a dorsal intercalary, originating as it does from an accumulation of perichordal sheath cells like the basidorsal. Its late development would account for the fact that it is directly converted into bone and its position at the posterior edge of the developing centrum is not inconsistent with homology with a dorsal intercalary.

The vertebral column of the goldfish shows a condition intermediate

between that of the cod, in which the arcualia are all mesenchymatous, and that of the herring, in which they are all cartilaginous; the dorsal arch rudiments being cartilaginous and the ventral arch rudiments mesenchymatous (as in the rudd and the pike). Only in the first five vertebrae do cartilaginous basiventrals occur, a form of recapitulation due to the development of the Weberian ossicles.

The presence of the Weberian apparatus is always correlated with considerable modification in the anterior vertebrae of Ostariophysi which has been described in great detail in the majority of genera, in particular by Bridge and Haddon (1890, 1892, 1893), by Chranilov (1926, 1927, 1929*a, b*) and by Adams (1928); and there has been much speculation, based mainly on comparative anatomy, as to their homologies.

Above the first vertebra lies a mass of cartilage which extends backwards to the intervertebral region between the third and fourth vertebrae. Matveiev considers it to be formed by the fusion of the first three interspinous bones with the neural spines of the first three vertebrae. Nusbaum considers it to be a part of the skull. Over this point I am in partial agreement with Matveiev and in total disagreement with Nusbaum. My sections showed clearly that there is no connexion between this structure and the skull and that it is contributed to by the basidorsals of the second, third and fourth vertebrae. The interspinous bones probably also contribute to it since there is no trace of them elsewhere. As has been shown, it gives rise to the neural arches and neural spines of the second, third and fourth vertebrae in the adult stage. I cannot agree with Matveiev when he states that the first vertebra takes part in the formation of this cartilage mass, my sections showing clearly that the basidorsal of the first vertebra forms part of the scaphium.

In the 11 mm. stage the spinal cord is surrounded in the region of the first vertebra by a peculiar ring of cartilage. Matveiev is the only author to refer to this structure but is unable to say what it represents. I have been able to show that it is probably an extension of the exoccipitals.

The homologies of the so-called "transverse processes" of the first and second vertebrae have aroused a great deal of controversy. Matveiev discusses them at considerable length but does not come to any definite conclusion about them. They are certainly not transverse processes or diapophyses since they proceed from the centrum while true diapophyses proceed from the neural arch and are in any case more characteristic of Tetrapoda than of Teleostei. I am of opinion that they are dorsal ribs since their origin and structure in the larva is similar to that of the dorsal or upper ribs of the pike and some other teleosts.

Matveiev makes no reference to the fusion of the second and third centra to form the "compound" vertebra. In the oldest stage which he studied the fusion had not yet commenced. Bloch (1900, 1916), however, in describing the anterior vertebrae of the loach (*Nemachilus barbatulus*) refers to the fact that the second and third vertebrae are inseparably fused. There is no doubt that in the goldfish the apparent second vertebra of the adult is formed by the fusion of the second and third centra during ontogeny.

Much doubt has been cast by earlier investigators on the homologies of the ribs of the fourth vertebra. Many have considered them to be transverse processes, presumably since they are more firmly attached to the centrum than the ribs of the posterior vertebrae and are shorter and stouter. Sections of the adult and of larval goldfish show that both in structure and development these processes are homologous with the pleural ribs of the posterior vertebrae.

The ossa suspensoria are believed by Bloch (1916), Sörenson (1890, 1895) and Wright (1884, 1885) to be modified transverse processes (i.e. parapophyses) while Weber, Muller, Nusbaum, Sagemehl and Chranilov believe them to be ribs. Since true ventral ribs have already been shown to be present in this vertebra it is obvious that the ossa suspensoria cannot represent ribs. Nor do the parapophyses take part in the formation of the ossa suspensoria which are clearly homologous with the haemapophyses found in other vertebrae of the goldfish. This was also found to be the case by Matveiev in the rudd.

With respect to the mode of development of the Weberian ossicles, three theories have been put forward. Weber (1820), Treviranus, Saagman Mulder, Baer and Breschet and the older authors considered these ossicles to be homologous with the ear ossicles of mammals. St Hilaire (1824), Muller (1843), Beaudelot (1868), Sagemehl (1884*a, b*, 1891), Bridge and Haddon (1893), Sachs (1912), Nusbaum (1908*a, b*), Thilo (1908), Chranilov (1929*a, b*), Grassi (1883) and Sidoriak (1898) believed the ossicles to be entirely derived from the anterior vertebrae. Sörenson, Bloch, Wright, Reis (1905) and Matveiev have held that the ossicles are derived in part from the ossification of ligaments and in part from the anterior vertebrae.

The development of the Weberian apparatus is a process of considerably greater complexity than was supposed by the earlier authors. From the time of its discovery by Weber in 1820 until the beginning of the present century, no one had investigated the appearance of the Weberian ossicles in the young stages of the Ostariophysi. It was tacitly assumed that the Weberian ossicles originated by the simple detachment of portions of the anterior vertebrae, and it was left to the comparative anatomist to decide

the exact relationships of the different structures. As with the function, so with the development, until recently all theories were based on morphology only, which, as Bridge has remarked, is a singularly inaccurate guide, although an essential basis, for embryological and physiological theories.

Nusbaum, in 1908, working on the common carp (*Cyprinus carpio*), was the first to undertake a real embryological study of the Weberian apparatus, and no further work was carried out in this direction until 1929, when Matveiev published a detailed account of the Weberian ossicles and anterior vertebrae in the young stages of the rudd (*Scardinius erythrophthalmus*).

The claustrum or "einlager" has aroused considerable diversity of opinion as to its origin. Grassi and Sagemehl believed it to be derived from the skull; Beaudelot and Sörenson considered it to be derived from the interspinous bone of the first vertebra; Wright and Bloch considered that it was derived from the supradorsal of the first vertebra; while Kindred believed it to be an intercalated cartilage.

I am in agreement with Kindred (1919) in believing the claustrum to be an intercalated structure. Kindred, however, states that in the horned pout (*Amiurus nebulosus*) the claustrum is of cartilaginous origin whereas in the goldfish I find it to be mesenchymatous.

The majority of investigators are agreed that the scaphium, so-called "stapes" or "deckel", represents a modified part of the neural arch of the first vertebra. In the goldfish, however, the scaphium clearly arises from two rudiments—the basidorsal of the first vertebra and an independent accumulation of mesenchyme, the former giving rise to the articulating and ascending processes, the latter to the concha stapedis. Matveiev attributes a similar dual origin to the scaphium in the rudd.

By most investigators the intercalarium, so-called "incus" or "lenker", is attributed to the neural arch of the second vertebra. Sörenson, however, considers it to be derived from the rib of the second vertebra, while Matveiev believes that it is formed by the fusion of an independent mesenchymatous rudiment with the neural arch of the second vertebra. In the goldfish the intercalarium arises from an independent ossification formed in the inter-ossicular ligament which fuses with part of the basidorsal of the second vertebra. The former gives rise to the manubrium incudis, the latter to the articulating and ascending processes.

The origin of the tripus, so-called "malleus" or "hebel" has, like that of the claustrum, aroused considerable diversity of opinion. Sagemehl, Bridge and Haddon, Bloch, Reis and Sachs believe it to be derived from the rib of the third vertebra, while Grassi attributes it to the transverse process of the third vertebra. Sörenson holds that it is derived from the rib of the

third vertebra together with an ossified ligament. Wright believes that it represents the rib of the third vertebra together with an ossification of the outer wall of the anterior chamber of the air-bladder. Muller and Nusbaum hold that it is derived from the rib of the third vertebra together with its transverse process (parapophysis), while Matveiev attributes it to the fusion of an ossified ligament lying between the lower anterior arches together with the ribs of the second and third vertebrae and the transverse process of the third vertebra.

The development of the tripus in the goldfish does not lend complete support to the theory of any previous investigator. The bulk of the tripus rudiment is formed by the fusion of the basiventral of the third vertebra with a distal rod-like mesenchymatous extension thereof; in other words by the parapophysis and pleural rib of the third vertebra, the former giving rise to the articulating process, the latter to the transformator process. Uniting the articulating and transformator processes is a mass of mesenchyme which has not been described by any previous investigator. Of structures like the "transverse processes" of the first and second vertebrae there is no trace in the third vertebra. The transverse processes referred to by Grassi, Muller and Nusbaum are the parapophyses. I cannot agree with Matveiev when he states that the second vertebra contributes to the tripus, an opinion in which he is alone. In the later stages (15-25 mm.) the transformator process is contributed to by an ossification in the outer wall of the anterior chamber of the air-bladder, while the anterior process is contributed to by an ossification in the interossicular ligament similar to that which forms the manubrium incudis. The tripus, therefore, is an ossicle of complex origin.

Previously it has been considered that the indirect system of the Weberian apparatus which is found in the Cyprinoidea (in which the intercalarium articulates with the vertebral column and the tripus possesses a transformator process which reverses the direction of motion of the chain) is more primitive than the direct system which is found in Siluroidea (in which the intercalarium does not articulate with the vertebral column and the tripus lacks a transformator process, so that the direction of motion of the chain is in the same sense as the movements of the wall of the air-bladder). The direct system has been considered as a degenerate form of the indirect system. The mode of development in the goldfish, however, is not consistent with such a theory. The condition of the intercalarium rudiment and the tripus rudiment in the young stages of the goldfish in particular, and presumably of the Cyprinoidea in general, closely resembles the condition of the intercalarium and tripus in many adult Siluroidea. It is therefore reasonable



to suppose that the direct system of the Weberian apparatus is more primitive than the indirect system and that the latter has evolved from the former.

Apart from these facts the development of the Weberian ossicles in the goldfish throws but little light on the development of the Weberian apparatus in time, since the apparatus has become specialized and it first appears very early in ontogeny. In all probability a study of its developmental history in some of the more primitive Ostariophysi, in particular the Characinae, the condition of the anterior vertebrae of which is much less abnormal, would throw more light on the question.

It is, perhaps, not out of place to add a few words on the function of these ossicles. Of the various theories put forward, recent experimental work has amply confirmed the one which postulates that they are a mechanism for the reception of vibrations. H. M. Evans (1925), working on several species of Cyprinidae, has shown that the method of attachment of the air-bladder to the tripus is such as to render it a peculiarly delicate mechanism for the purpose of recording rapidly recurring movements of small amplitude. The tip of the transformer process of each tripus is attached anteriorly to the ossa suspensoria by a small triangular muscle consisting of unstriated fibres and known as the tensor tripodis, posteriorly to the outer wall of the air-bladder. The system of ossicles is thus a rigid whole held in place by the tensor tripodis, which immediately pulls the tip

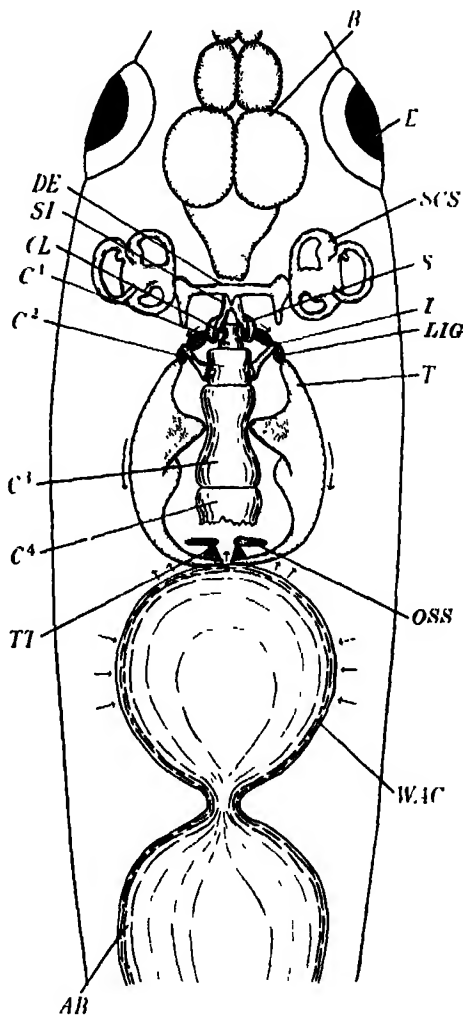


FIG. 8. Diagram to show the relationship between the air-bladder, the Weberian ossicles and the internal ear (modified from Chramlov.)

of the tripus back into its original position after movement of the air-bladder wall has caused it to be displaced. Vibrations are therefore transmitted by changes of tension, not, as in the auditory ossicles of mammals, by impact.

A necessary condition for the successful reception of vibrations by the anterior part of the air-bladder (termed by Chranilov the "Weberian air-chamber") would be that the pressure of the contained gas should be kept at a certain uniform level. The ductus communicans between the Weberian air-chamber and the air-bladder proper is innervated by a special branch of the vagus nerve which forms a complex plexus surrounding the ductus. Also surrounding the ductus, and connected with the nerve plexus, is a powerful sphincter muscle. The nerve plexus receives afferent fibres from the walls of the posterior sac, and Evans' experiments have shown that change of pressure in the posterior sac causes immediate reflex closing of the sphincter. Hence the pressure in the Weberian air-chamber is maintained at a uniform level which enables it to respond to vibrations. The ganglion and sphincters function as a mechanism for maintaining a uniform pressure in the Weberian air-chamber, a conclusion which has been fully borne out by later experiments by Evans and Damant (1928).

While Evans has shown that the Weberian apparatus could, in fact, function as an effective mechanism for the transmission of vibrations from the air-chamber to the inner ear, it is to Kuiper (1915) and von Frisch (1936) that we are indebted for demonstration of the fact that it does indeed so act. The experiments of Kuiper have shown that destruction of the Weberian apparatus results in fish of the order Ostariophysi ceasing to respond to any extent to vibrations, while continuing to respond to other types of stimuli. Von Frisch has demonstrated experimentally that the sacculus and lagena of the ear in Cyprinidae and Siluridae is specially adapted for the reception of sound vibrations conveyed to it by the Weberian chain, and that both range of hearing and ability to discern tones is highly developed. His experiments proved, not only that the Ostariophysi have a sensitivity to sound stimuli little inferior to that of man, but also that they are much superior in this respect to those fish which lack a Weberian apparatus.

I wish to acknowledge my very great indebtedness to Professor E. W. MacBride, not only for suggesting the subject of this research, but also for constant encouragement and advice. My thanks are also due to Assistant-Professor H. R. Hewer and Dr J. R. Norman for criticism and suggestions, to Mr T. L. Green for help in regard to technique, and to Mr L. Haig for his assistance in rearing the fish.

### SUMMARY

The early stages of the development and formation of the anterior vertebrae and Weberian ossicles in the goldfish (*Carassius auratus*) are described from the time of hatching till the adult condition is acquired at 25 mm. and it is concluded that:

(1) The spinal cord in the region of the first vertebra (which lacks a neural arch) is partly enclosed by a backward extension of the exoccipitals.

(2) The basidorsals of the second (part only), third and fourth vertebrae fuse with the first three interspinous bones to form a massive arch of cartilage which later gives rise to the neural arches and spines of the compound vertebra and the fourth vertebra.

(3) The "transverse processes" of the first and second vertebrae are dorsal ribs.

(4) The centra of the second and third vertebrae fuse to form a compound centrum.

(5) The ossa suspensoria are modified haemapophyses.

(6) The claustrum arises as a direct ossification of the connective tissue forming the wall of the atrium sinus imparis.

(7) The scaphium arises in part from the basidorsal of the first vertebra, in part from an independent mesenchymatous rudiment.

(8) The intercalarium arises in part from the basidorsal of the second vertebra and in part from an ossification in the interossicular ligament.

(9) The tripus arises in part from the basiventral of the third vertebra and in part from an independent mesenchymatous rudiment, together with a small ossification of the interossicular ligament and a small ossification of the outer coat of the air-bladder.

(10) The condition of the embryonic Weberian chain in the goldfish is so similar to the adult condition in the Siluroidea that in all probability the indirect cyprinoid system has evolved from the direct siluroid system.

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## ABBREVIATIONS

<i>AB.</i>	Air-bladder.	<i>IN.</i>	Internoural bone.
<i>AB. EXT.</i>	Outer coat of the air-bladder.	<i>LIG.</i>	Interossicular ligament.
<i>AB. INT.</i>	Inner coat of the air-bladder.	<i>MI.</i>	Manubrium incedis.
<i>AO.</i>	Aorta.	<i>MY.</i>	Myotome.
<i>B.</i>	Brain.	<i>NCH.</i>	Notochord.
<i>BD.</i>	Basidorsal.	<i>NS.</i>	Neural spine.
<i>BV.</i>	Basiventral.	<i>OSS.</i>	Os suspensorium.
<i>C.</i>	Centrum.	<i>PBO.</i>	Pharyngeal process of basi- occipital.
<i>CA.</i>	Cartilage arch.	<i>Post. Zyg.</i>	Posterior zygapophysis.
<i>Cart. R.</i>	Cartilage ring.	<i>PS.</i>	Perichordal sheath.
<i>CL.</i>	Claustrum.	<i>R.</i>	Rib (pleural).
<i>CM.</i>	Cartilage mass.	<i>S.</i>	Scaphum.
<i>COE.</i>	Coelem.	<i>SC.</i>	Spinal cord.
<i>CR.</i>	Cranium.	<i>SCS.</i>	Semicircular canals.
<i>CS.</i>	Concha stapedis.	<i>SG.</i>	Spinal ganglion.
<i>DE.</i>	Ductus endolymphaticus	<i>SPV.</i>	Saccus paravertebralis
<i>E.</i>	Eye.	<i>T.</i>	Tripus.
<i>FCP.</i>	Fibrocartilage pad.	<i>TR.</i>	Transformator process.
<i>FS.</i>	Fibrous sheath.	<i>TP.</i>	Transverse process.
<i>HK.</i>	Head kidney.	<i>TT.</i>	Tensor tripodis.
<i>HMP.</i>	Haemapophysis.	<i>WAC.</i>	Weberian air-chamber.
<i>I.</i>	Intercalarium.		

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## Low temperature and insect activity

BY KENNETH MELLANBY\*

*The University of Sheffield*

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### INTRODUCTION

Insects are cold blooded, and their metabolism and activity is very greatly influenced by the temperature of their bodies, which temperature is almost entirely dependent on that of the surrounding environment. A low temperature inhibits activity, and a higher temperature usually stimulates the animal. The range over which any species can survive is limited above and below by lethal temperatures, and within this range lies the much narrower zone of normal activity. Most insects can for instance survive exposure to a much lower temperature than that at which activity ceases. Certain terms used for conciseness in this paper require definition. The "chill-coma temperature" (Belehradek 1935) is the temperature at which the insect is immobilized by the cold. The "cold-death point" is the temperature below which exposure is lethal.

It appears that if there is one single factor more than any other which controls the distribution of an insect, it is the temperature below which activity never normally takes place. Insect distribution and survival is no doubt greatly affected by such factors as lethal high and low temperatures and unfavourable atmospheric humidity, but if, in any region, the temperature does not rise sufficiently often above that at which the normal activity of a species begins, that species will cease to exist although all other conditions are favourable to life. In temperate regions climatic conditions are seldom sufficiently extreme actually to kill many insects, but numerous species are unable to live actively or breed successfully there.

The importance of the temperature below which activity never occurs has been realized by previous workers, but they have mostly made the mistake of assuming that an insect under natural conditions will still exhibit activity until the temperature falls to that at which chill-coma

\* Sorby Research Fellow of the Royal Society.

occurs. This is far from being the case, for the lowest temperature at which some form of activity is possible frequently appears to be several degrees below that at which spontaneous movements take place. For instance, it has previously been shown that the tsetse fly, *Glossina palpalis*, is able to fly at 14° C, but it never appears spontaneously active under natural conditions below 20° C, and a hungry fly at (say) 18° C will die from starvation without moving to find food (Mellanby 1936).

Previous workers have not only assumed that the low temperature at which activity in the laboratory begins is the same as that at which normal spontaneous movements start, but they also failed to realize how greatly the immediate past history of an insect affects the position of the chill-coma temperature. As will be shown below, unless we know under what conditions an insect has been living during the day previous to the experiment, measurements of the chill-coma temperature may be of little significance.

In the course of this work, some observations on the lethal effects of low temperatures were made, and as these results indicate that the cold-death point is related to the chill-coma temperature they have been included in this paper.

It is difficult to determine satisfactorily in the laboratory the precise temperature at which spontaneous movements begin. Unless a good deal is known about the behaviour of the insect in the field, quite erroneous results may be obtained. All the experiments I have made indicate that spontaneous movements do not occur until the temperature is considerably above that at which activity becomes possible, but even so the unnatural conditions often caused the insects to move at temperatures lower than those at which movements begin in the field. In this connexion I propose to give details of results obtained only in experiments which satisfactorily simulate the natural conditions.

Experiments have been made to determine the effect of different conditions of temperature on the behaviour of insects when subjected to a new thermal environment; more specifically an attempt was made to ascertain whether previous exposure to cool surroundings would produce a degree of acclimatization which would enable the insect to exist more easily at low temperatures than if it had previously been kept in a warm environment. The results indicate that though the chill-coma temperature and the cold-death point are considerably affected, it is doubtful whether the activity of the insect will be greatly influenced.



## INSECTS USED IN THE EXPERIMENTS

The following species were used:

The cockroach, *Blatta orientalis* L. [Orthoptera]. Adults and nymphs all gave similar results.

The bed-bug, *Cimex lectularius* L. [Hemiptera]. Adults.

*Rhodnius prolixus* Stål [Hemiptera]. Adults.

The greenbottle fly, *Lucilia sericata* Meigen [Diptera]. Larvae and adults.

The bluebottle fly, *Calliphora erythrocephala* Macquart [Diptera]. Larvae and adults.

The tsetse fly, *Glossina palpalis* (Robineau-Desvoidy) [Diptera]. Adults.

This work has been going on for some years, and many thousands of individual insects have been under observation. The final results obtained, using improved techniques, have been obtained with smaller numbers. For the chill-coma determinations (see Table I) in the latest experiments at least 20 insects were used to fix each point. In some cases (e.g. *Blatta*) over 500 individuals were used.

## THE CHILL-COMA TEMPERATURE

To determine the chill-coma temperature several different types of apparatus were tried, but eventually the simplest was found the most satisfactory. This consisted of a glass Erlenmeyer flask of 250 c.c. capacity placed inside a wide-mouthed ("quart" size) vacuum jar. The flask was plugged with cotton-wool, and the temperature inside measured with a quick-reading thermometer. The bottom of the flask was made rough by a layer of sand fastened in wax, so that the insects could get a grip. The temperature was controlled by placing water in the vacuum jar and adding iced or warm water to alter the temperature. Temperatures below zero were obtained by adding ice and salt. It was soon found easy to obtain any required temperature and maintain it for a considerable period. On some occasions it was convenient to place the insects in large cages or other containers exposed to the outside winter temperatures. The chill-coma temperature as measured with the insects outside or in the more restricted laboratory conditions was found to be the same in all cases. The humidity was not controlled, but the air in contact with the insects was always approaching saturation. In the field, insects immobilized by low temperatures will also normally be exposed to almost saturated air.

Most previous workers on the chill-coma temperature have either cooled the insects slowly until they have become immobilized (Bertram 1935) or they have first put them at a low temperature which caused immobility and then slowly warmed them, noting the point at which activity began (Krogerus 1932). Both methods were tried in these experiments, but erratic results were obtained depending considerably on the rate of cooling and warming. Later, the insects were transferred directly to some particular low temperature, which was maintained for at least 15 min., during which period the behaviour of the insects was observed. This method gave consistent results for comparable material. In some ways it made the work more tedious, as fresh insects had to be used at each temperature, but it was possible roughly to plot out the areas of activity using small numbers, then with larger numbers at the critical temperatures to obtain the final results.

A considerable difference between the chill-coma temperature and that which allows full normal activity has been noted in certain cases by previous workers (Krogerus 1932; Bertram 1935). Using the technique described above, it appeared that the majority of the insects tested were able to show full activity about 1° C above chill-coma temperature. These were non-flying insects. Flying insects are mostly unable to fly until a point is reached many degrees above the chill-coma temperature, although they can crawl actively much below the flight temperature. If flying insects in a cage are stimulated as the temperature is reduced, it will be seen that a point is soon reached where, although they can still fly, they are unable to produce sufficient power (the cold has reduced their metabolic rate) to fly upwards, and like an aeroplane suffering from engine trouble they lose height. They are still able to crawl when they cannot fly at all but fall vertically through the air.

The insects used were bred in the laboratory. They were kept at various different temperatures, and transferred direct from these temperatures down to the vicinity of the chill-coma point. It was soon noticed that insects of the same species taken from different temperatures possessed different chill-coma temperatures. The warmer the conditions under which it had been kept, the higher the chill-coma temperature for each species (Table I). The results in the table are not strictly speaking the chill-coma temperatures, they are approximately 1° C higher—that is, they are the lowest temperatures at which the insects were active. Though the position of the chill-coma temperature for the adults of *Lucilia* and *Calliphora* was considerably affected by previous conditions, the temperature at which flight became possible was not so affected. With *Lucilia* flight was possible

at 14° C whatever conditions the fly had previously sustained. It is interesting to note that this temperature (i.e. 14° C) is 9° C above the chill-coma temperature for flies acclimatized to 30° C, and 12° C above that for those from 15° C. A similar comparison can be made with *Calliphora*, which can fly at as low a temperature as 9° C. These observations support the view that previous exposures to different temperatures, though affecting considerably the chill-coma temperature, have little effect on metabolism in the zone of normal activity (see p. 483 below). *Lucilia* or *Calliphora* are able to fly when their metabolic rates reach a high enough level, and the position of the temperature at which their metabolism reaches that level appears to be fixed.

TABLE I. THE LOWEST TEMPERATURES (° C) AT WHICH ACTIVITY IS POSSIBLE IN A NUMBER OF INSECTS WHICH HAVE BEEN PREVIOUSLY EXPOSED TO DIFFERENT TEMPERATURES

Species of insect	Insects previously exposed to		
	14-17° C	30° C	36° C
<i>Blatta orientalis</i>	2.0	7.5	9.5
<i>Cimex lectularius</i>	4.5	7.0	7.5
<i>Rhodnius prolixus</i>	8.6	10.5	12.0
<i>Lucilia sericata</i> :			
(a) Larva	2.0	4.5	-
(b) Adult (crawling)	3.5	6.0	
<i>Calliphora erythrocephala</i> :			
(a) Larva	1.0	4.0	
(b) Adult (crawling)	1.0	5.4	

An examination of Table I will show that very different ideas as to the possible geographical distribution of the various species might be obtained depending upon the temperature at which the insects had been kept in the laboratory prior to the experiment.

#### THE PERIOD OF ACCLIMATIZATION

"Acclimatization" will be used here to describe the alteration of the chill-coma temperature by previous exposure of the insects to high and low temperatures. For instance, a cockroach kept at 30° C and then transferred to 15° C will be said to be acclimatized to 15° C when its chill-coma temperature becomes lower than 2° C, and if the insect is then returned to 30° C it will become acclimatized to this latter temperature when it is unable to crawl normally below 7.5° C (see Table I).

Acclimatization is not a long process. With one exception, all the insects mentioned in Table I become fully acclimatized in less than 20 hr. The exception is the cockroach; though specimens kept at 15° C become acclimatized to 30° C within 20 hr., others previously kept at 30° C do not become fully acclimatized to 15° C for 2-3 days. In this latter case, partial acclimatization takes place much more quickly.

Very short exposures of only 5 min. to new temperatures were found not to have any acclimatizing effect, but after 2 hr. a temperature was found to have had some influence. Exposures of up to 12 hr. produced partial acclimatization in all species, but the results were somewhat erratic, and it was found most convenient to allow an exposure of at least 20 hr. and so obtain complete acclimatization. Once an insect is completely acclimatized the period for which it is exposed to that temperature appears to have no effect on the speed at which it can become acclimatized to another. Thus bed-bugs bred for several generations at 30° C became acclimatized to 15° C as rapidly as did others which had only been kept at 30° C for 24 hr.

The rapidity of acclimatization accounts for the erratic results obtained when trying to find the chill-coma temperature by slowly reducing the temperature of the insect. If it comes from warm conditions, and the temperature is reduced slowly, the creature may be partly acclimatized to cooler conditions before it is finally immobilized. The slower the reduction in temperature the greater the acclimatization and so the lower the chill-coma temperature.

No acclimatization appears to occur when an insect is in a state of chill-coma. Thus cockroaches from 30° C were kept for 5 days at between 2 and 3° C, during which period they remained completely immobilized (though other individuals from 15° C were able to crawl normally all the time at this lower temperature). If any of the immobilized cockroaches were warmed to 15° C they recovered after some time (see p. 479 below), but an immediate determination of their chill-coma temperature showed that it was the same as when they left 30° C and had not been affected by the sojourn at 2-3° C. Similarly, bed-bugs from 30° C remained immobilized at 5° C for many hours, and only became acclimatized, so that their chill-coma temperature rose, at 9.5° C or above. It seems that this type of acclimatization only takes place in insects capable of movement, and not in individuals cooled below their chill-coma temperature.

## THE RATE OF RECOVERY FROM CHILL-COMA

If an insect is cooled exactly to the chill-coma temperature, and then warmed slightly, it recovers within a few seconds, even if it has been kept immobilized for a considerable period. But if an insect is cooled several degrees below the chill-coma temperature, recovery is a much slower process. When insects are immobilized at any particular low temperature and then transferred to warmer conditions, their rate of recovery is largely affected by their previous acclimatization. This is brought out by the results given in Table II. Cockroaches previously kept at 15° C were cooled at 1° C, which is practically their chill-coma point; on returning to 15° C they recovered at once. Others acclimatized to 30° C were also cooled to 1° C, but in their case this was about 5° C below the chill-coma point, which accounts for the long time they took to recover when transferred to 15° C. The same phenomenon was observed with all the other species used in these experiments.

TABLE II. TIME TAKEN BY COCKROACHES (*BLATTA ORIENTALIS*)  
TO RECOVER FROM EXPOSURE TO LOW TEMPERATURE

Temp. of exposure ° C	Period of exposure hr.	Time taken to recover after transference from low temperature at 15° C	
		Acclimatized to 15° C	Acclimatized to 30° C
1	24	Under 1 min.	1½-2½ hr.
1	72	Under 1 min.	1½-2½ hr.
-3.0	1	12 min.	2-2½ hr.

Insects in chill-coma recover more quickly when transferred to a high temperature than they do when transferred to one not far above the chill-coma point. Thus one batch of cockroaches recovered in 90 min. at 15° C and in 15 min. at 35° C. Bed-bugs previously acclimatized to 30° C and immobilized at 3.5° C recovered in 2 hr. at 13° C and within 1 min. at 35° C. The body temperature of a bed-bug will reach that of its surroundings within 5 min.; the slow recovery at 13° C is not due to the body temperature being too low to allow activity, but, presumably, to the slowness of the internal recovery processes.

The differences in rate of recovery depending on the extent to which the insect has been chilled and on the position of the recovery temperature means that attempts to find the chill-coma temperature by slowly warming immobilized insects (see Krogerus 1932) is unlikely to give accurate results unless the rate of warming is extremely slow. If warming is quicker than 4 hr. for a rise of 1° C the temperature may rise too high before the slow

recovery processes at a low temperature are complete. This method has the advantage over that of slowly cooling until immobility is produced, that if rapidly immobilized the insect retains the properties of the acclimatization it possessed before the experiment started (see p. 478 above).

#### THE COLD-DEATH POINT

There seems to be some relation between the chill-coma temperature, the time required to recover from chill-coma, and the cold-death point. I have been unable for technical reasons to make a complete study of cold resistance in the insects concerned in this paper, but the following experiments with the cockroach appear significant.

Temperatures below zero were obtained inside the cooling unit of a refrigerator in the place where the ice trays normally lie. It was possible to obtain temperatures down to  $-9^{\circ}\text{C}$  and to maintain them within a constancy of  $1^{\circ}\text{C}$  for periods up to 3 hr. The insects were exposed inside  $3 \times 1$  in. glass tubes; the open end of each tube was covered with gauze. Results of exposures to various temperatures are given in Table III.

TABLE III. DEATH AND SURVIVAL OF COCKROACHES (*BLATTA ORIENTALIS*) AFTER EXPOSURE TO TEMPERATURES BELOW ZERO ('ENTIGRADE. SIX INDIVIDUALS IN EACH EXPERIMENT

Temp. of exposure $^{\circ}\text{C}$	Period of exposure hr.	Cockroaches previously kept at	
		$15^{\circ}\text{C}$	$30^{\circ}\text{C}$
$-3.0$	$1\frac{1}{2}$	S	S
$-5.5$	1	S	D
$-5.5$	9	S	D
$-6.8$	$1\frac{1}{2}$	S	D
$-8.0$	1	5D, 1S	D
Between $-4$ and $-8$	15	D	D

S = survived, D = dead.

It will be seen that cockroaches from  $15^{\circ}\text{C}$  are considerably more "cold-hardy" than those from  $30^{\circ}\text{C}$ . Acclimatization with regard to cold-hardiness is as rapid as it is to chill-coma (see p. 478 above). After 20 hr. at  $30^{\circ}\text{C}$  cockroaches previously kept at  $15^{\circ}\text{C}$  were killed in 1 hr. at  $-5.5^{\circ}\text{C}$ , whereas others kept at  $15^{\circ}\text{C}$  until immediately before the experiment survived this and even lower temperatures. Cockroaches from  $30^{\circ}\text{C}$  became acclimatized to the conditions obtaining at  $15^{\circ}\text{C}$  within 20 hr., and became almost as cold-resistant as others kept at  $15^{\circ}\text{C}$  for long periods.

These experiments (Table III) seem to indicate that once an insect is acclimatized the temperature at which death occurs from freezing is fairly definite, and within limits its position is not greatly affected by the length of the exposure. Thus cockroaches from 15° C all survived either 1 or 9 hr. at -5.5° C, but only 1 hr. at -8° C killed most of the individuals. Death may also occur after prolonged exposure to temperatures above zero (Uvarov 1931; Belehradek 1935). It appears likely that at a temperature where some insects from a higher temperature are in chill-coma and some from a lower are not, those that are not immobilized will survive longest. Cockroaches from 15° C certainly survived at temperatures between 2 and 5° C for 3 weeks; none died out of forty individuals, and they were able to crawl during the whole exposure. Other cockroaches from 30° C showed some mortality when exposed to this temperature for only 5 days; two individuals out of eight never recovered when they were returned to a higher temperature. These numbers are much too small to determine the point with certainty, but they do suggest that the temperature where death occurs after long chilling (as opposed to freezing) may be altered by previously keeping the insects at different temperatures.

#### THE THRESHOLD TEMPERATURE FOR SPONTANEOUS MOVEMENTS

There is only one really satisfactory method of discovering the threshold temperature for spontaneous movement; that is by observing the behaviour of the insect under natural conditions in its normal habitat. Provided that the periodicity of the activity is undisturbed, and the temperature of the exact spot occupied by the insect is measured, then observations over a period including all types of weather may give an accurate result. It is important to measure the temperature in the spot the insect occupied *before* it began its activity—if it rests in a burrow the stimulating conditions must be those in the burrow and not those in the open outside, even if the insect is actually caught in the open. Both temperature and humidity may be quite different in two places near to one another (see Buxton 1932; Mellanby 1933).

The most satisfactory laboratory experiments were performed using the bed-bug. As this insect does not fly, and normally lives in crevices and restricted spaces, it is possible to produce conditions not grossly unnatural. Also recent work has shown that in a natural infestation activity occurs at definite times (Mellanby 1939), which suggests when observations should be made in the experiments. Finally, the bed-bug feeds infrequently, particularly at low temperatures, so that the insect is

likely to be in a similar physiological state at the beginning and end of an experiment. Insects which feed frequently are difficult to work with, as their state of hunger changes so rapidly and introduces further variables.

The technique adopted was simple. Adult bugs were fed and kept until they reached the stage at which they are known to be most active in a natural infestation (see Mellanby 1939). The bugs were then placed in glass tubes containing crumpled blotting paper to a depth of 2 cm. The tubes were placed for several hours at the experimental temperature, and then two more pieces of folded paper were added, each filling the tube a further 2 cm. If such a tube containing six bugs were left overnight at 23° C, in the morning the bugs would be found distributed over the three pieces of paper. After a further 24 hr. they would be found to have again rearranged themselves. This method does not indicate whether a bug has moved and gone back to its original resting place, but the clear-cut difference between the results obtained above and below the "threshold" temperature, and the fact that most of the activity was nocturnal (as in a natural infestation) suggests that this is a satisfactory method. It may sometimes cause movements to be missed, but any movement noted appears to be really spontaneous. The bugs were in the dark except for about 1 min. each morning and evening when they were examined; this brief illumination did not appear to disturb the insects.

Experimental temperatures between 0 and 15° C were obtained using the different chambers of a Williams's incubator; the temperatures were not constant and fluctuated a few degrees depending on the outside conditions and the amount of ice in the apparatus, but they never fluctuated more than 1° C between two observations. Tubes were also placed in different positions in a refrigerator, where a similar range of temperatures was obtained.

Several series of experiments were made, and their results may be stated simply. In one experiment, in which four tubes were examined twice a day for a month, no movements were noted below 11° C. Between 11 and 12° C movements were noted on 6% of the occasions, between 12 and 13° C on 46%, and above 13° C on 80% of the nights. In the other experiments very few movements were observed below 12° C, and above that temperature movements were frequent. Incidentally, care must be taken not to use very hungry bugs, as though these are never active at the lower temperatures they appear to remain quite still even at higher temperatures except in the presence of food. This is presumably connected with the fact that such bugs, though able to survive for a long time in a state of inactivity, die soon after movement unless they are fed (Mellanby 1938). The various



experiments indicate that the threshold for spontaneous movement is in the region of 12° C. It was confirmed that bed-bugs which never moved when kept for weeks between 6 and 10° C were able to crawl normally at that temperature if stimulated.

No satisfactory experiments were devised for the other insects. In some cases (e.g. *Rhodnius*) insufficient was known about the natural behaviour. All these insects appeared to be able to move when stimulated at temperatures several degrees below those at which they moved spontaneously, but it was not possible to show satisfactorily that under unnatural conditions the insects were not always moving below their true threshold temperatures for spontaneous movement.

Some results obtained with the tsetse fly, *Glossina palpalis*, may be mentioned here. The tsetse, like the bed-bug, feeds at intervals of several days, and so may be observed over periods of many hours without its hunger stage altering greatly. In the laboratory, in an experiment attempting to simulate natural conditions, the tsetse never flew to seek for food below 20° C, though it was able to fly at 14° C. Observations made in the field showed also that the insects never appeared until the temperature among the bush in which they were lurking rose above 20° C.

There is one interesting fact about these observations on the bed-bug and the tsetse. In the bed-bug the developmental zero is not much below 13° C (Jones 1930; Mellanby 1935), though movements are possible at much lower temperatures. With the tsetse also the developmental zero is not far from the threshold for spontaneous movements. The developmental zero for the larva of *Lucilia sericata* is about 9° C (Cousin 1929), which is several degrees above the chill-coma temperature. From these observations I am inclined to suggest that though insects may be able to move at temperatures considerably below their developmental zero, they seldom do so spontaneously. There may perhaps be some physiological connexion between the two points, but it will require further investigation before a definite connexion can be shown.

#### THE RATE OF MOVEMENT WITHIN THE ZONE OF NORMAL ACTIVITY

In many cold-blooded animals the rate of movement often appears to be a measure of the rate of metabolism. At each particular temperature above the chill-coma point all healthy adult bugs move at approximately the same speed, and within limits the higher the temperature the higher the speed. There are, of course, individual variations, and weak individuals move erratically, but on the whole the behaviour is very consistent. To

measure the speed of movement, the bugs were placed on blotting paper illuminated from above (within limits the intensity of illumination did not affect the insect's behaviour). The bugs always moved under these conditions, and the distance they traversed was indicated by following the insect with a pencil while the time was recorded on a stop-watch. The average speed at 15° C was found to be 59 cm./min., and at 25° C it was 208 cm./min. The maximum speed of 255 cm./min. is reached at 32° C—above this temperature the bug may move very rapidly for a short distance, but crawling does not appear "normal". If a bug is transferred from 30 to 15° C for a few seconds it runs rapidly and erratically, and then it settles down to the characteristic speed for the new temperature. After being kept at 15° C for several hours, the bug's speed is the same as after half a minute. Transference from 15 to 30° C gives a similar result. Provided that a bug has been kept above the chill-coma point and below any high temperature which would cause damage, the speed of movement (and probably the rate of metabolism also) reaches the equilibrium for that particular temperature very soon. In this, the bed-bug is behaving much as does a marine *Amoeba* (Pantin 1924). If, however, a bug in chill-coma is placed at a higher temperature, it may take a considerable time to recover at all, and even longer before it runs at the normal speed. Below 10° C the speed depends on the temperature previously experienced. A bug from 15° C will run as fast as 10 cm./min. at 8° C, but one transferred to 8 from 36° C will be only just above its chill-coma temperature and will crawl very slowly indeed (under 2 cm./min.).

Exposure to warm conditions for a long period may cause a bed-bug to exhaust its reserves, and then it may behave differently when transferred to another temperature compared with a bug from cooler conditions. But it will be the effects of hunger and not those of the change in temperature which are acting. Provided the physiological hunger stage is the same, within the zone of normal activity the rate of movement at any particular temperature is not altered by previous experiences.

## DISCUSSION

The position of the chill-coma temperature has been shown not to be a fixed point, but to be altered by the temperature at which the insect has been previously kept. Bodenheimer and Klein (1930) found that the "biological zero for creeping" of the ant, *Messor semirufus*, in Palestine alters during the different seasons. It does not seem possible to correlate the changes with the temperatures measured (mean minimum temperature,

etc.), but it is quite possible that the conditions to which the insects were actually subjected just previous to the experiments may account for the differences. Other workers have found that the chill-coma temperature for different animals (e.g. Coelenterates, Mayer 1914) is not always the same, but they have usually considered the alteration was seasonal or due to geographical causes, and have not realized that it might have been produced easily and rapidly in the laboratory.

There has been much work on cold-hardiness in insects, and on their metabolism below zero (Payne 1926; Kalabuchov 1935; Kozhantshikov 1938; Salt 1936, etc.). Payne has shown that certain oak-borer larvae (Synchronidae, Pyrochroidae, Elateridae, Cerambycidae) are more hardy in winter than in summer. She has also shown that desiccation will lower the freezing point of insects. The present work is in agreement with that of most other workers, except that it now appears that the degree of cold-hardiness of an insect may be altered more rapidly and easily than was previously realized.

The temperature under which an insect lives alters the position of the chill-coma temperature and the cold-death point, but I do not think that the threshold temperature for spontaneous movement is commonly affected. Acclimatization takes place too rapidly. At temperatures near the spontaneous movement threshold all the insects studied were almost completely acclimatized within 20 hr.; at these temperatures metabolism is slow, and any differences during the period of acclimatization are unlikely to affect the economy of the animals. It appears probable that the threshold temperature for spontaneous movement will be the most constantly fixed point in the lower range of an insect's experience.

The physiological changes which accompany the alterations in chill-coma temperature, etc., are not understood; they will form the subject of future investigation. Some of the results fit in with Belehradek's (1935) theory that the viscosity of the protoplasm may be altered, but the theory will require further modification to account for them all.

In the laboratory, with insects kept under constant conditions, definite chill-coma temperatures and cold-death points can be found. In the field, where the temperature is all the time fluctuating, the position of these points will fluctuate also. When the day is warm, the insect will be more easily immobilized by cold and more easily killed by freezing. When the day is cool, the reverse will be the case. Whether or not these changes will affect the economy of the species depends on the speed of acclimatization and the rapidity with which the temperature changes.

## SUMMARY

1. The "chill-coma temperature" for five species of insects belonging to three different orders was found to be considerably affected by the temperature at which they had been living. Individuals of the same species from warm conditions were immobilized at higher temperatures than those from cool.

2. Acclimatization with respect to the chill-coma temperature usually occurred within 20 hr.

3. When insects were cooled below the chill-coma temperature and then returned to warm conditions, the length of time taken to recover was longer the further the creatures were cooled below the chill-coma point.

4. During chill-coma, insects from high temperatures did not become acclimatized to the low temperature.

5. Insects of the same species from warm conditions were less cold-hardy, and more easily killed by exposure to conditions below zero, than those from cool.

6. The threshold temperature for spontaneous movements was usually several degrees higher than that at which movements were possible. There was some indication that the threshold is in the region of the developmental zero for the species.

7. Within the zone of normal activity, when an insect was transferred from one temperature to another, its rate of movement quickly altered to that characteristic of the new temperature. It is only outside the zone of normal activity that the rate of movement was greatly affected by the previous conditions. The rate of movement may be controlled directly by the rate of metabolism.

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## The genetical analysis of a sex-limited character in *Drosophila melanogaster* and its bearing on the evolution of secondary sexual characteristics

BY CECIL GORDON<sup>1</sup> AND FRANCES GORDON

*Department of Biometry, University College, London, and the  
Department of Natural History, University of Aberdeen*

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### INTRODUCTION

Sex-limited characters may be studied from three points of view in so far as the object of enquiry may be to elucidate (*a*) the nature of the *genetic difference* involved, (*b*) the developmental process which eventually leads to somatic sex differences or to differences of behaviour, and (*c*) the way in which the genetic difference arises and is established as the basis of a *secondary sexual difference* in the course of evolution.

This investigation deals chiefly with the first and third issues stated above. Three different types of genetic difference might be expected to give rise to sex-limited characters, and in fact all three have been found to exist. One occurs when genes whose effects are incompletely dominant and additive are located on the X-chromosome. This is the "dosage" effect described by Muller (1932). A second is the specific effect of Y-borne genes illustrated by "bobbed" in *Drosophila* (Stern 1927). The third is when the sex-limited character depends directly or indirectly upon the genetic

<sup>1</sup> Carnegie Teaching Fellow in the University of Aberdeen.

difference which is also responsible for the primary distinction between the sexes, or is determined by a gene balance of the same general type. Some of the colour pattern genes in *Lebistes* are probably of this type (Winge 1927).

Sex-limited characters may arise by single gene mutations, by a series of gene mutations which reinforce one another, or by the addition or loss of chromosomes. Conceivably such differences may be established by two different types of genetic selection which Darwin distinguished as Natural Selection and Sexual Selection. The first possibility implies that the mutations which originally exhibited the sex-limited differences were conserved by virtue of greater individual *fitness* capable of being expressed in terms of viability and fertility. The second possibility implies a form of *assortative mating*. In so far as sexual selection contributes to species characteristics which are not sex-limited it implies preference of similar phenotypes for one another. In so far as it contributes to secondary sexual characteristics of a species it implies preference of unlike phenotypes for one another. The mathematical analysis of genetic selection has shown that the efficacy of both assortative mating and of natural selection depends on (a) the number of genes involved, and (b) whether the genes involved are autosomal, X-borne or Y-borne.

Greater fitness in the sense defined above may be conferred on both sexes by genes with an alternative somatic effect which is limited by the internal environment or by the gene complex characteristic of one or the other sex. Genes with alternative somatic effects might also accumulate by virtue of their specific effect on the viability of the female sex, if the same male may fertilize a large number of females, or if, as in some Arthropods, the female reproductive phase is relatively protracted and the contribution of the male is restricted to a relatively small fraction of her reproductive life. The production of secondary sexual characteristics by natural selection can only be interpreted as an incidental consequence of alternative somatic effects of the same genes. Conversely, while sexual selection implies the pre-existence of other secondary sexual characteristics and may be instrumental in producing new ones, it may have a far wider significance as a means of concentrating specific differences which are not sex-limited.

The stage at which the differentiation of the sex-limited characters occurs has no relevance to a discussion of sexual selection except when, as happens in some crustacea, such differences only emerge after mating has taken place. On the other hand, the efficacy of natural selection will depend on whether differentiation occurs at an early or late stage in the development of the individual. Very early differentiation is consistent with

the intensive consequences of what Haldane (1924) calls *familial selection*. On the other hand, retardation of sexual differentiation till the onset of the sexually adult stage, as in mammals and birds, diminishes the intensity of natural selection, especially if the effective reproductive phase is relatively short. Clearly, sex-limited characters confined to the post adult phase (e.g. in *Lerneæ*) cannot be established by sexual selection alone.

Till recently the importance of Darwin's theory of sexual selection suffered an eclipse as a result of two sorts of criticisms to which it had been subjected. The first was a salutary reaction against gratuitous speculations unsupported by experimental evidence about the role of sexual preference in animal behaviour. With experimental technique it is now possible to study the role of sexual preference as an experimental issue, and the importance of sexual selection, or of other forms of assortative mating, as the means whereby specific differences may be established now receives a more sympathetic hearing. The other objection was the difficulty of reconciling the operation of selection on one sex with the fact that inheritance is common to both. This was due to belief in blending inheritance. Modern knowledge of particulate inheritance shows that unisexual, though less intense than bisexual, selection, can lead to the same results in the end.

Experimental analysis of the genetic basis of sex-limited characters and the effects of selection upon them has hitherto been obstructed by the circumstance that most known sex-limited characters are highly stable, and incipient characters which depend on the combined action of many genes are difficult to detect or to elucidate.

This communication deals with a sex-limited character in *Drosophila melanogaster*.

#### MATERIALS AND METHOD

In the course of mapping new genes, detected by inbreeding, in *D. melanogaster*, a Vple stock (black-purple-vestigial-arc-speck) was used as a second chromosome standard. In the segregating cultures a difference of palp coloration was observed. This was given the name "Brown Palp". It was found to be limited to the female. High-grade Brown Palp females were selected as parents and the character reappeared only among their female offspring.

The following matings were then made: (a) selected Brown Palp females were mated to males from the same culture (Brown Palp ♀ × "Brown Palp" ♂); (b) selected Brown Palp females were mated to males from an Oregon pure line (Brown Palp ♀ × Oregon ♂); (c) unselected males from the

Brown Palp stock were mated with Oregon females (Oregon ♀ × "Brown Palp" ♂). These results are given in Table I, which gives actual numbers and the numerical values of two ratios "*p*" and "*e*" corresponding respectively to the proportion of Brown Palp females in the total female population, and the proportion of high grade types in the Brown Palp class. The values of *p* and *e* for the offspring of Brown Palp ♀ × "Brown Palp" ♂ are greater than the corresponding values for the offspring from the other crosses. At this stage we may interpret the differences recorded by saying that Oregon behaved as a "Non-Brown-Palp" stock and that Brown Palp is an incompletely dominant character. We shall see later that this statement needs to be qualified in the light of subsequent observations. The data also show that although Brown Palp is suppressed in the males, it can be transmitted by them to their offspring. The difference between the results from crosses Brown Palp ♀ × Oregon ♂ and Oregon ♀ × Brown Palp ♂ is probably due to the fact that the females were selected for Brown Palp from a genetically mixed population, while the males were necessarily unselected. Subsequent experiments showed that males and females from genetically homogeneous Brown Palp stocks were equally capable of transmitting the character to their female offspring.

TABLE I. MATING BETWEEN BROWN PALP AND OREGON STOCKS

Mating	Males		Females		" <i>p</i> "	" <i>e</i> "
	Non-Brown Palp	Non-Brown Palp	Brown Palp low grade	Brown Palp high grade		
Brown Palp ♀ × "Brown Palp" ♂	235	63	117	108	0.78	0.48
Brown Palp ♀ × Oregon ♂	214	85	95	38	0.61	0.28
Oregon ♀ × "Brown Palp" ♂	216	130	86	16	0.42	0.15

$$"p" ("penetrance") = \frac{\text{Total Brown Palp females}}{\text{All females}}$$

$$"e" ("expressivity") = \frac{\text{High-grade Brown Palp females}}{\text{Total Brown Palp females}}$$

(The terms "penetrance" and "expressivity" are strictly only applicable to homozygous stocks, but in this instance the calculation of *p* and *e* are analogous to the calculation of penetrance and expressivity *sensu stricto*.)

A preliminary experiment showed that an analysis of the genetic mechanism was possible. Before proceeding further a genetically homogeneous stock was established by about 40 generations of brother-sister



mating. Thereafter experiments were undertaken (a) to elucidate the genetic nature of differences between females, (b) to explore the mechanism of suppression in the males.

#### THE ANALYSIS OF DIFFERENCES BETWEEN FEMALES

In these experiments methods of scoring and culturing were refined, because the character was found to be more difficult to detect in older animals. Flies which were clearly classifiable as Brown Palp on emergence would be scarcely recognizable as such after the lapse of a few days. To eliminate errors arising from this age difference, care was taken to score all animals within 2 hr. of emergence. This was done by culturing the animals on Offermann's dead yeast medium which ensures a burst of emergences 10 days after setting up. To concentrate attention on newly emerged flies the cultures were kept at about 4° C after emergence had started. In the mornings they were put into an incubator at 26° C and examined every 2 hr. For reasons of personal convenience the interval between the beginning of emergence and transference to the lower temperature varied somewhat. Since the investigation involved discrimination of different *grades* the animals were very carefully orientated with regard to the source of illumination while they were scored. The females were divided into four classes. Oregon males were used as a standard for class 0, and females from the Brown Palp pure line for class 3. It is a matter of general experience that shade differences must be scored very rapidly, owing to fatigue. Concentration on individual animals leads to blurring. Those flies which would normally pass as Non-Brown Palp, but which gave rise to doubts were placed in class 1. Animals definitely, but not maximally, Brown Palp were placed in class 2. There were frequent misgivings concerning the scoring, but the system led to consistent results.

For each group of flies an index of phenotypic intensity based on the use of arbitrary weights for the several grades was calculated as follows: Let the number of flies in class 0 be  $a$ , in class 1 be  $b$ , in class 2 be  $c$ , in class 3 be  $d$ , the value of the index is then given by:  $\frac{b + 2c + 3d}{a + b + c + d}$ . Its numerical limits are 0 and 3.0. Owing to visual fatigue the method outlined here is only applicable where there are sharp contrasts, i.e. in segregating cultures. For instance it is not possible to score the slight variations in the Oregon, Brown Palp and *Star-Curly-Dichaete* ( $S/Cy$ ,  $D/+$ ) (*vide infra*) stocks or among offspring of Oregon by  $S/Cy$ ,  $D/+$ , even though the females were slightly darker than the males. Differences between indices

should be interpreted with caution, as a difference of say 0.5 at the level 0 would be more difficult to establish than one at the level 2. Moreover, the same gene substitution may have a different quantitative effect at different levels. The results were not submitted to statistical analysis. In view of the arbitrary nature of the scoring, this would lead to a spurious appearance of an order of accuracy not justified by the material.

The method of tracking down the effect of each chromosome was the one first developed by Altenburg and Muller (1920) in the analysis of "Truncate", and subsequently extended by the Morgan school to other characters. It consists essentially of crossing the stocks to be analysed to standard stocks whose chromosomes are "marked" by carrying a *dominant* gene or by virtue of the sex of the individual parent. The method is illustrated by the accompanying diagram which illustrates the *Star-Curly-Dichaete* ( $S/Cy, D/+$ )  $\times$  Brown Palp cross (fig. 1). The second chromosome markers are *Star* ( $S$ ), a roughened eye character, and *Curly* ( $Cy$ ), a turned-up wing. The third chromosome is marked by only one character, *Dichaete* ( $D$ ), shortened bristles and outstretched wings. The symbol  $E$  is used for chromosomes of *Star-Curly-Dichaete* stock origin without the three marker genes, and is therefore applicable either to the  $X$  or to the third chromosome which does not carry the *Dichaete* gene. The fourth chromosome has been ignored. The nomenclature adopted is stated in Table II. At this

TABLE II

Stock	Males			Females		
	$\frac{E}{Y}$	$\frac{S}{Cy}$	$\frac{D}{E}$	$\frac{E}{E}$	$\frac{S}{Cy}$	$\frac{D}{E}$
$S/Cy, D/+$						
Brown Palp	$\frac{Bp}{Y}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$
Oregon	$\frac{Or}{Y}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$

stage in the investigation there was no available evidence to show whether the Brown Palp effect is due to one or more genes on one and the same chromosome or to genes located on different chromosomes. The symbolism employed in Table II and fig. 1 is a deviation from the normal practice in

so far as the formula  $\frac{Bp}{Bp}$  assigned to any one of the chromosome pairs of inbred Brown Palp stock signifies this fact alone, and should not be interpreted to mean that the chromosome specified carries one or more specific genes which contribute to the observed character differences. In sub-

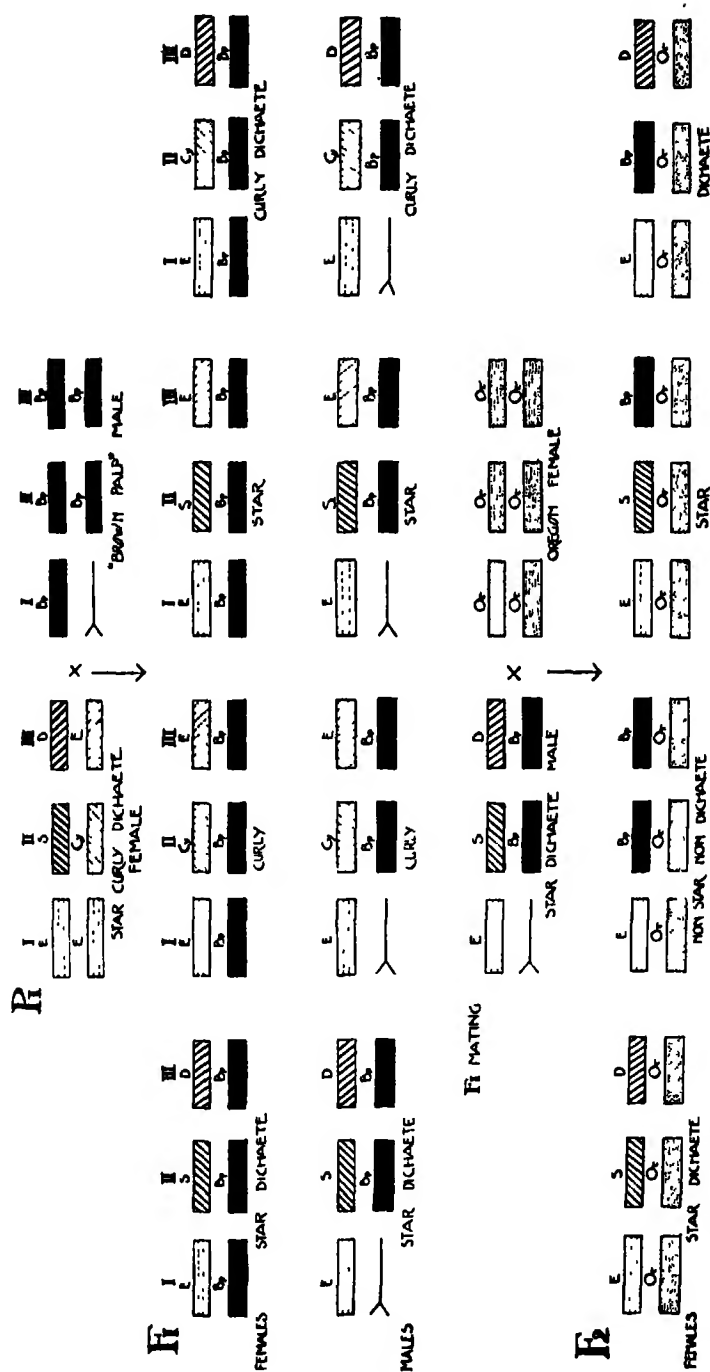


FIG. 1. Schema showing crosses between Brown Palp stock, males and females and individuals from stocks heterozygous for the dominant gene *Star*, *Curl* and *Dichaeete*. Since these genes are dominant the phenotypes which carry them exhibit the mutant character.

sequent descriptions numerical symbols for the chromosomes of *Drosophila* will not be used. It will be understood that when comparing effects due to substituting *Bp* for *S*, we are dealing with a second chromosome substitution, or that when comparing effects due to substituting *Bp* for *D* we are dealing with a third chromosome substitution.

#### CONSISTENCY OF RESULTS

Table III gives a comparison between flies of the same genetic constitution. It is summarized from Tables VI–XIII, which show that there is no overlap between groups *a* and *b* on the one hand, and groups *c* and *d* on the other. These roughly correspond to heterozygous and homozygous Brown Palp. It will be noted that after crosses with marker stocks “re-constituted” females with the same chromosome make-up as the original Brown Palp stock yielded numerical values of 2.32–3.00 for the index of

TABLE III

	Genotypes	Table ref. no.	Index
(a)	$\frac{E}{Or} \quad \frac{Bp}{Or} \quad \frac{Bp}{Or}$	VIa	1.96
		VIb	1.64
		VIII	2.00
(b)	$\frac{Bp}{Or} \quad \frac{Bp}{Or} \quad \frac{Bp}{Or}$	Xa	1.84
		Xb	1.76
		XII	1.88
(c)	$\frac{E}{Bp} \quad \frac{Bp}{Bp} \quad \frac{Bp}{Bp}$	VIIa	2.94
		VIIb	3.00
		IX	2.52
(d)	$\frac{Bp}{Bp} \quad \frac{Bp}{Bp} \quad \frac{Bp}{Bp}$	XIa	3.00
		XIb	2.76
		XIII	2.32

phenotypic intensity. Though the numerical value of the phenotypic index for a homogeneous Brown Palp stock cannot be greater than 3.0, it is in fact difficult to assess how far short of this limit an estimate based on the method of scoring described might be. However, when sibs of segregating cultures are compared the difficulties described are avoided. In view of the difficulties of scoring most of the values obtained were as near to the maximum as may be expected. The lowest values (2.52 and 2.32) are from cultures listed in Tables IX and XIII. They occur in segregations which have low indices throughout. Possibly this was due to

environmental conditions such as variation of length of time in the incubator and ice box. It is also possible that a personal error crept in, because both the cultures were scored at a time when no other cultures were being similarly treated.

## THE GENETIC BASIS OF DIFFERENCES BETWEEN FEMALES

The initial crosses were

(a) *S/Cy*, *D*/ + ♀ × Brown Palp ♂

$$\begin{pmatrix} E & S & D \\ \bar{E} & \bar{C}_y & \bar{E} \end{pmatrix} \times \begin{pmatrix} Bp & Bp & Bp \\ \bar{Y} & \bar{Bp} & \bar{Bp} \end{pmatrix},$$

(b) *S/Cy*, *D*/ + ♂ × Brown Palp ♀

$$\left( \frac{E}{Y} \quad \frac{S}{Cy} \quad \frac{D}{E} \times \frac{Bp}{Bp} \quad \frac{Bp}{Bp} \quad \frac{Bp}{Bp} \right).$$

The results from these two matings are given in Tables IV and V. There is no difference between the genetic constitution of the corresponding classes of female offspring. The genetic constitution of the males differs with respect to the X-chromosome. Thus the Star Dichaete males from Table IV have the constitution  $\frac{E}{Y} \frac{S}{\bar{B}p} \frac{D}{Bp}$ , and those from Table V  $\frac{Bp}{Y} \frac{S}{\bar{B}p} \frac{D}{\bar{B}p}$ . The males were crossed to Brown Palp and to Oregon females.

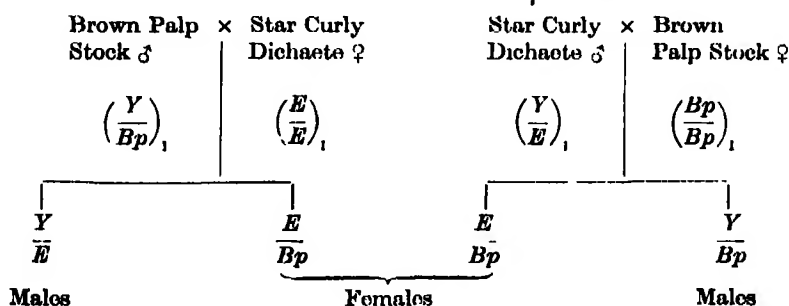


FIG. 2. To illustrate possible existence of two relevant genotypic male classes in reciprocal crosses, on the assumption that the *X*-chromosome contributes to the Brown Palp effect.

Two advantages of using marker *males* are: (a) the absence of crossing over, (b) the possible (fig. 2) existence of two relevant genotypic classes of offspring. The Star Dichaeete males from cultures listed in Table IV were crossed to Oregon and Brown Palp females (Tables VIa, VIb and VIIa,

VIIb respectively). In an analogous experiment Curly Dichaete males were substituted for Star Dichaete males (Tables VIII and IX). The Star Dichaete males from cultures listed in Table V were crossed to Oregon and Brown Palp females (Tables Xa, Xb and XIa, XIb respectively), and again the Curly Dichaete males were substituted for the Star Dichaete males (Tables XII and XIII). It should be noted that of the total of 2107 males all were maximally pale.

TABLE IV

 $S/Cy, D/+ \delta \times Bp \text{ } \bar{q}$ 

Females	Genotype			Phenotype	$\frac{E}{E} \frac{S}{Cy} \frac{D}{E} \times \frac{Y}{Bp} \frac{Bp}{Bp} \frac{Bp}{Bp}$				Index
					0	1	2	3	
(i)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	Star Dichaete	8	55	22	0	1.16
(ii)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{E}{Bp}$	Star	32	52	10	0	0.78
(iii)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{D}{Bp}$	Curly Dichaete	46	44	2	0	0.52
(iv)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{E}{Bp}$	Curly	66	47	2	0	0.44
	Total females				152	198	36	0	0.70
	Total males				242	0	0	0	0.00

TABLE V

 $S/Cy, D/+ \delta \times Bp \text{ } \bar{q}$ 

Females	Genotype			Phenotype	$\frac{E}{Y} \frac{S}{Cy} \frac{D}{E} \times \frac{Bp}{Bp} \frac{Bp}{Bp} \frac{Bp}{Bp}$				Index
					0	1	2	3	
(i)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	Star Dichaete	13	29	12	0	0.98
(ii)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{E}{Bp}$	Star	16	33	12	0	0.93
(iii)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{D}{Bp}$	Curly Dichaete	18	22	0	0	0.55
(iv)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{E}{Bp}$	Curly	30	15	0	0	0.33
	Total females				77	99	24	0	0.73
	Total males				133	0	0	0	0.00

TABLE VI

		$\frac{Y}{E}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	$\times$	$\frac{Or}{Or}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$		
		(a)								
Females	Genotype	Phenotype			0	1	2	3	Index	
(i)	$\frac{E}{Or} \frac{S}{Or} \frac{D}{Or}$	Star Dichaete			37	12	3	0	0.35	
(ii)	$\frac{E}{Or} \frac{S}{Or} \frac{Bp}{Or}$	Star			1	8	45	14	2.05	
(iii)	$\frac{E}{Or} \frac{Bp}{Or} \frac{D}{Or}$	Dichaete			46	14	3	0	0.33	
(iv)	$\frac{E}{Or} \frac{Bp}{Or} \frac{Bp}{Or}$	Non-Star			0	11	35	9	1.96	
		Non-Dichaete								
		Total females			84	45	86	23	1.20	
		Total males			208	0	0	0	0.00	
		(b)								
(i)	$\frac{E}{Or} \frac{S}{Or} \frac{D}{Or}$	Star Dichaete			37	36	7	0	0.63	
(ii)	$\frac{E}{Or} \frac{S}{Or} \frac{Bp}{Or}$	Star			1	13	50	7	1.86	
(iii)	$\frac{E}{Or} \frac{Bp}{Or} \frac{D}{Or}$	Dichaete			39	22	1	0	0.39	
(iv)	$\frac{E}{Or} \frac{Bp}{Or} \frac{Bp}{Or}$	Non-Star			2	26	36	5	1.64	
		Non-Dichaete								
		Total females			79	97	94	12	1.14	
		Total males			258	0	0	0	0.00	

TABLE VII

		$\frac{Y}{E}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	$\times$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$			
		(a)									
Females	Genotype	Phenotype			0	1	2	3	Index		
(i)	$\frac{E}{Bp} \frac{S}{Bp} \frac{D}{Bp}$	Star Dichaete			4	9	4	0	1.00		
(ii)	$\frac{E}{Bp} \frac{S}{Bp} \frac{Bp}{Bp}$	Star			0	0	0	15	3.00		
(iii)	$\frac{E}{Bp} \frac{Bp}{Bp} \frac{D}{Bp}$	Dichaete			4	3	4	1	1.17		
(iv)	$\frac{E}{Bp} \frac{Bp}{Bp} \frac{Bp}{Bp}$	Non-Star			0	0	1	16	2.94		
		Non-Dichaete									
		Total females			8	12	9	32	2.03		
		Total males			89	0	0	0	0.00		

TABLE VII (*continued*)

Females	Genotype			Phenotype	0	1	2	3	Index
	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$						
(i)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	Star Dichaete	12	15	10	2	1.05
(ii)	$\frac{E}{Bp}$	$\frac{S}{Bp}$	$\frac{Bp}{Bp}$	Star	0	0	3	30	2.91
(iii)	$\frac{E}{Bp}$	$\frac{Bp}{Bp}$	$\frac{D}{Bp}$	Dichaete	7	9	7	0	1.00
(iv)	$\frac{E}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	Non-Star Non-Dichaete	0	0	1	30	3.00
				Total females	19	24	21	62	2.00
				Total males	90	0	0	0	0.00

TABLE VIII

Females	Genotype			Phenotype	0	1	2	3	Index
	$\frac{Y}{E}$	$\frac{Cy}{Bp}$	$\frac{D}{Bp} \times \frac{Or}{Or}$						
(i)	$\frac{E}{Or}$	$\frac{Cy}{Or}$	$\frac{D}{Or}$	Curly Dichaete	64	1	0	0	0.02
(ii)	$\frac{E}{Or}$	$\frac{Cy}{Or}$	$\frac{Bp}{Or}$	Curly	8	21	17	0	1.20
(iii)	$\frac{E}{Or}$	$\frac{Bp}{Or}$	$\frac{D}{Or}$	Dichaete	21	17	0	0	0.45
(iv)	$\frac{E}{Or}$	$\frac{Bp}{Or}$	$\frac{Bp}{Or}$	Non-Curly Non-Dichaete	1	9	34	10	2.00
				Total females	94	48	51	10	0.89
				Total males	119	0	0	0	0.00

TABLE IX

Females	Genotype			Phenotype	0	1	2	3	Index
	$\frac{Y}{E}$	$\frac{Cy}{Bp}$	$\frac{D}{Bp} \times \frac{Bp}{Bp}$						
(i)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{D}{Bp}$	Curly Dichaete	66	4	0	0	0.05
(ii)	$\frac{E}{Bp}$	$\frac{Cy}{Bp}$	$\frac{Bp}{Bp}$	Curly	6	27	10	0	1.07
(iii)	$\frac{E}{Bp}$	$\frac{Bp}{Bp}$	$\frac{D}{Bp}$	Dichaete	21	47	11	0	0.87
(iv)	$\frac{E}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	Non-Curly Non-Dichaete	0	2	18	26	2.52
				Total females	93	80	39	21	0.95
				Total males	113	0	0	0	0.00



TABLE X

		$\frac{Y}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	$\times$	$\frac{Or}{Or}$	$\frac{Or}{Or}$	$\frac{Or}{Or}$			
		(a)									
Females	Genotype	Phenotype			0	1	2	3	Index		
(i)	$\frac{Bp}{Or} \frac{S}{Or} \frac{D}{Or}$	Star Dichaeto			39	22	1	0	0.39		
(ii)	$\frac{Bp}{Or} \frac{S}{Or} \frac{Bp}{Or}$	Star			0	6	43	12	2.10		
(iii)	$\frac{Bp}{Or} \frac{Bp}{Or} \frac{D}{Or}$	Dichaeto			37	19	0	0	0.34		
(iv)	$\frac{Bp}{Or} \frac{Bp}{Or} \frac{Bp}{Or}$	Non-Star			0	14	45	4	1.84		
		Non-Dichaete									
		Total females			76	61	89	16	1.18		
		Total males			212	0	0	0	0.00		
		(b)									
(i)	$\frac{Bp}{Or} \frac{S}{Or} \frac{D}{Or}$	Star Dichaeto			29	32	11	0	0.75		
(ii)	$\frac{Bp}{Or} \frac{S}{Or} \frac{Bp}{Or}$	Star			0	14	57	19	2.06		
(iii)	$\frac{Bp}{Or} \frac{Bp}{Or} \frac{D}{Or}$	Dichaeto			35	34	7	0	0.63		
(iv)	$\frac{Bp}{Or} \frac{Bp}{Or} \frac{Bp}{Or}$	Non-Star			0	20	44	4	1.76		
		Non-Dichaeto									
		Total females			64	100	119	23	1.33		
		Total males			252	0	0	0	0.00		

TABLE XI

		$\frac{Y}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	$\times$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$			
		(a)									
Females	Genotype	Phenotype			0	1	2	3	Index		
(i)	$\frac{Bp}{Bp} \frac{S}{Bp} \frac{D}{Bp}$	Star Dichaeto			8	14	14	0	1.17		
(ii)	$\frac{Bp}{Bp} \frac{S}{Bp} \frac{Bp}{Bp}$	Star			0	0	0	34	3.00		
(iii)	$\frac{Bp}{Bp} \frac{Bp}{Bp} \frac{D}{Bp}$	Dichaete			7	23	7	0	1.00		
(iv)	$\frac{Bp}{Bp} \frac{Bp}{Bp} \frac{Bp}{Bp}$	Non-Star			0	0	0	29	3.00		
		Non-Dichaete									
		Total females			15	37	21	63	1.97		
		Total males			169	0	0	0	0.00		

(b)

Females	Genotype			Phenotype	0	1	2	3	Index
(i)	$\frac{Bp}{Bp}$	$\frac{S}{Bp}$	$\frac{D}{Bp}$	Star Dichaete	5	15	22	2	1.48
(ii)	$\frac{Bp}{Bp}$	$\frac{S}{Bp}$	$\frac{Bp}{Bp}$	Star	0	0	7	29	2.81
(iii)	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{D}{Bp}$	Dichaete	10	15	15	0	1.12
(iv)	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	$\frac{Bp}{Bp}$	Non-Star	0	0	5	16	2.76
				Non-Dichaete					
				Total females	15	30	49	47	1.91
				Total males	97	0	0	0	0.00

$$\frac{Y}{Bp} \quad \frac{Cy}{Bp} \quad \frac{D}{Bp} \quad \times \quad \frac{Or}{Or} \quad \frac{Or}{Or} \quad \frac{Or}{Or}$$

Females	Genotype			Phenotype	0	1	2	3	Index
(i)	$\frac{Bp}{Or}$	$\frac{Cy}{Or}$	$\frac{D}{Or}$	Curly Dichæte	44	1	0	0	0.02
(ii)	$\frac{Bp}{Or}$	$\frac{Cy}{Or}$	$\frac{Bp}{Or}$	Curly	8	15	8	0	1.00
(iii)	$\frac{Bp}{Or}$	$\frac{Bp}{Or}$	$\frac{D}{Or}$	Dichæte	17	20	2	0	0.62
(iv)	$\frac{Bp}{Or}$	$\frac{Bp}{Or}$	$\frac{Bp}{Or}$	Non-Curly Non-Dichæte	0	13	46	5	1.88
	Total females				69	49	56	5	0.93
	Total males				---	---	---	---	---

$$\begin{array}{ccc} Y & Cy & D \\ \overline{Bp} & \overline{Bp} & \overline{Bp} \end{array} \times \begin{array}{ccc} Bp & Bp & Bp \\ \overline{Bp} & \overline{Bp} & \overline{Bp} \end{array}$$

Females	Genotype	Phenotype	0	1	2	3	Index
(i)	$\frac{Bp}{Bp} \frac{Cy}{Bp} \frac{D}{Bp}$	Curly Dichaeete	36	5	0	0	0.12
(ii)	$\frac{Bp}{Bp} \frac{Cy}{Bp} \frac{Bp}{Bp}$	Curly	8	23	4	0	0.89
(iii)	$\frac{Bp}{Bp} \frac{Bp}{Bp} \frac{D}{Bp}$	Dichaeete	11	22	10	0	0.98
(iv)	$\frac{Bp}{Bp} \frac{Bp}{Bp} \frac{Bp}{Bp}$	Non-Curly	1	3	25	21	2.32
		Non-Dichaeete					
		Total females	56	53	39	21	1.15
		Total males	—	—	—	—	—

## EFFECT OF THE X-CHROMOSOME

Hitherto no evidence has been submitted to show whether the gene or genes which contribute to the Brown Palp effect are located on one or more chromosomes, and from what has gone before we cannot infer that any particular chromosome carries such genes. As already stated the symbol  $Bp/Y$  does not signify that the  $X$ -chromosome makes a specific contribution to the effect. It merely labels the origin of this chromosome from a genetically homogeneous stock for the  $Bp$  chromosome. The experimental evidence embodied in the foregoing (Tables VI–XIII) will now be examined to decide whether the  $X$ -chromosome makes any specific contribution to the Brown Palp effect. We have seen that the males from cultures listed in Table IV differ from those listed in Table V, with respect to their  $X$ -chromosome. Hence their female offspring will show whether the substitution of  $E$  for its allelomorph on an  $X$ -chromosome derived from Brown Palp stock has any effect on the palp coloration. To avoid periphrasis we shall refer to this henceforth as the substitution of  $E$  for " $Bp$ ". Let us for example compare the females which segregated from the cultures respectively listed in Tables VI and X. The female parents of each were of Oregon stock. The males are phenotypically alike, namely Star-Dichaete, and differ with respect to the  $X$ -chromosome. The male parent in Table VI

TABLE XIV

Type of cross	Relation of $X$ -chromosomes	Table ref. no.	Index	Remarks
Star Dichaete $\times$ Oregon	$\frac{E}{Or}$	VIa	1.20	$Bp > E$
	$\frac{Bp}{Or}$	VIb	1.14	
	$\frac{E}{Or}$	Xa	1.18	
	$\frac{Bp}{Or}$	Xb	1.33	
Star Dichaete $\times$ Brown Palp	$\frac{E}{Bp}$	VIIa	2.03	$Bp < E$
	$\frac{Bp}{Bp}$	VIIb	2.00	
	$\frac{E}{Bp}$	XIa	1.97	
	$\frac{Bp}{Bp}$	XIb	1.91	
Curly Dichaete $\times$ Oregon	$\frac{E}{Or}$	VIII	0.89	$Bp > E$
	$\frac{Bp}{Or}$	XII	0.93	
	$\frac{E}{Bp}$	IX	0.95	
Curly Dichaete $\times$ Brown Palp	$\frac{Bp}{Bp}$	XIII	1.15	$Bp > E$
	$\frac{E}{Bp}$			

has an *E* X-chromosome, and the male parent in Table X has the *Bp* X-chromosome. With respect to the marker genes the phenotypic female offspring are alike. They differ genetically only in so far as their X-chromosomes are derived from different sources. For this reason the phenotypic index based on the totals for the two classes of female offspring gives us a means of estimating any significant contribution of X-borne genes. Similar comparisons may be based on Tables VII and XI, VIII and XII or IX and XIII. The indices for corresponding pairs are summarized in Table XIV. It will be seen that *Bp* is once less than *E* and three times greater than *E*. The maximum difference,  $1.15 - 0.95 = 0.20$ , is surprisingly small. Thus there is no clear evidence for any marked differences between the effects of the X-chromosomes, though it does not follow that all X-chromosomes have no effect on palp coloration.

#### EFFECT OF THE AUTOSOMES

Since we have already shown that the X-chromosome has no decisive effect it will be ignored in future discussion of results and the autosomes only will be specified. Hitherto we had assumed that genes which promote the expression of the Brown Palp character were only present in chromosomes derived from the Brown Palp stocks. An examination of Tables IV and V shows that the effect of substituting an *S* for a *Cy* second chromosome is to raise the Brown Palp index quite considerably. For example female offspring with the constitution  $\frac{S}{Bp} \frac{D}{Bp}$ , and female offspring with the constitution  $\frac{Cy}{Bp} \frac{D}{Bp}$  (Table IV(i) and (iii)) respectively yielded indices 1.16 and 0.52. This difference is representative of three other corresponding pairs recorded in Tables IV and V. This would imply that Brown Palp genes were present in the *S/Cy*, *D*/+ stock and that the *S*-chromosome carries more Brown Palp genes than the *Cy*-chromosome. A similar but less marked effect is produced by the substitution of *D* for *E*. Thus we may also say that the third chromosome of *S/Cy*, *D*/+ carried Brown Palp genes and that more Brown Palp genes are associated with the *Dichaete* gene than with its allelomorphs in these cultures. Comparisons which exhibit the effects of substituting second or third chromosomes of Brown Palp stock for corresponding chromosomes from the marker genes can also be extracted from the foregoing Tables. The relevant data for the substitution *Bp* for *Cy* are contained in Tables VIII, IX, XII and XIII, which show that it raises the index for all third chromosome groupings.

The minimum change in index is  $0.45 - 0.02 = 0.43$  (Table VIII) and the maximum change is  $2.32 - 0.89 = 1.43$  (Table XIII). As we have already seen the absolute values must be treated with caution, but we can say that the substitution of *Bp* for *Cy* always raises the index, and is always considerable in effect. Similarly, the effect of substituting *Bp* for *S* can be seen from Tables VI, VII, X and XI. In sixteen substitutions of *Bp* by *S* the index is raised thirteen times, lowered twice and on one occasion has no effect. This surprising result can only mean that the second chromosomes of the *S/Cy*, *D/+* stock with the marker gene *Star* makes at least as powerful a contribution to the Brown Palp effect as the second chromosome of the Brown Palp stock. The minimum difference is  $3.00 - 3.00 = 0$ , and the maximum difference is  $1.48 - 1.12 = 0.36$ , both from Table XI. On the other hand a second chromosome from *S/Cy*, *D/+* stock with the marker gene *Curly* makes a much less potent contribution to the Brown Palp character than a second chromosome from Brown Palp stock. The difference between *Bp* and *Cy* is therefore quite definite, whereas the difference between *S* and *Bp* may quite well be due to experimental error.

We now examine the third chromosome substitutions. Substituting *Bp* for *D* raises the index on all twenty-four occasions (Tables VII–XIII). The minimum is  $0.89 - 0.12 = 0.77$  from Table XIII, and the maximum is  $3.00 - 1.00 = 2.00$  from Tables VIIa and XIa. Thus we can also say that a third chromosome derived from Brown Palp stock is more effective in promoting the exhibition of the sex-limited character than is a third chromosome of *Star-Curly-Dichaete* stock with the marker gene *Dichaete*.

Results of the comparison of sibs thus show:

$S = > Bp \gg Cy$  for the second chromosome.

$Bp \gg D > E$  for the third chromosome.

So far the effects of Oregon autosomes have not been discussed. They have been used as a background against which the others may be discussed. Previous deductions have been made from an internal analysis of each table, i.e. by the comparison of sibs. In further analysis two important difficulties must be pointed out. The first is that comparisons must be made *between* (as opposed to *within*) segregating groups. The second is failure to obtain figures for certain types. Very early in the investigation a difference between the male and female palp coloration was noticed in almost all stocks. The palp coloration of the female was slightly darker than that of the male with the notable exception of the *D II*/Payne stock in which both males and females were dark. So slight was the difference in most stocks that independent confirmation was necessary in order to settle whether

there had been a real difference or merely a subjective expectation. In a few experiments colleagues were asked to select the darker of pairs of flies in which the heads only were visible. This invariably led to the selection of the females. Independent confirmation for the existence of an incipient palp colour difference in Non-Brown Palp stocks came later, when it was found that the *S* and *D* chromosomes were found to have more effect than the *Cy* and *E* chromosomes. This could account for the differences observed in the *S/Cy*, *D/+* stock. It is probable that chromosomes of the same strength with regard to Brown Palp genes as *Cy* and *E*, if associated with the latter, would give a female palp coloration indistinguishable from that of the males. It was not, however, possible to obtain a comparable figure for the *S/Cy*, *D/+* stock owing to lack of contrast. The same difficulty was encountered in dealing with the Oregon stock, and to a less extent with the offspring of *S/Cy*, *D/+* and Oregon matings. Thus the indices for  $\frac{S}{Cy} \frac{D}{E}$ ,  $\frac{Or}{Or}$ ,  $\frac{S}{Or} \frac{D}{Or}$ ,  $\frac{S}{Or} \frac{E}{Or}$ ,  $\frac{Cy}{Or} \frac{D}{Or}$ ,  $\frac{Cy}{Or} \frac{E}{Or}$  were not available, although the four latter might have been possible with elaborate counterchecking. However, this was not undertaken, because initial experiments had failed to give contrasts that could be scored. The phenotypic index values were, however, certainly greater than zero. In what now follows, comparisons must therefore be treated with considerable reserve, and deductions are not as reliable as those based on comparison of sibs.

TABLE XV

$\frac{Or}{Bp} \frac{Or}{Bp}$	Table no.	$\frac{Cy}{Bp} \frac{D}{Bp}$	Table no.	$\frac{Cy}{Bp} \frac{E}{Bp}$	Table no.	$\frac{S}{Bp} \frac{D}{Bp}$	Table no.	$\frac{S}{Bp} \frac{E}{Bp}$	Table no.	$\frac{Bp}{Bp} \frac{Bp}{Bp}$	Table no.
1.96	VI	0.52	IV	0.44	IV	1.16	IV	0.78	IV	2.94	VII
1.64	VI	0.55	V	0.33	V	0.98	V	0.93	V	3.00	VII
2.00	VIII									2.52	IX
1.84	X									3.00	XI
1.76	X									2.76	XI
1.88	XII									2.32	XIII

We cannot compare the effect of each Oregon autosome with its complement. We can, however, compare the effects of the two jointly with a number of alternative combinations. The relevant data are summarized in Table XV. We see that against a background of Brown Palp autosomes, the two Oregon autosomes are more effective than *Cy* + *D*, *Cy* + *E*, *S* + *D*, *S* + *E* but less effective than the two Brown Palp autosomes. Since we already know that *S* = > *Bp* > *Cy*, that *Bp* > *D* > *E*, and that the index of the Oregon stock is greater than 0, then *S* = > *Bp* > *Or* > *Cy* and *Bp* > *Or* > *E*,



males will be "reconstituted" "Brown Palp"  $XO$  males. About 250 of such Non-Ruby males were examined, and all were found to be maximally pale. Thus the absence of the  $Y$ -chromosome did not result in the appearance of Brown Palp males.

### *The $XXY$ females*

L. V. Morgan (1922) showed that when two  $X$ -chromosomes are attached  $X$ -borne characters can be inherited matrilineally and patrilineally. The females in such crosses are  $\widehat{XX}Y$ . These females get their  $\widehat{XX}$  from their mother and the  $Y$  from the father, while the male offspring get the  $X$  from the father and the  $Y$  from the mother. An  $\widehat{XX}Y$  stock carrying "Forked" on the  $X$ -chromosome was found to be Non-Brown Palp. Females from this stock were crossed with "Brown Palp" males.

$$\begin{array}{ccc}
 P_1 & \frac{\widehat{ff}}{Y} \frac{N}{\widehat{N}} \frac{N}{\widehat{N}} \text{ } \text{?} & \times \quad \frac{Bp}{Y} \frac{Bp}{\widehat{Bp}} \frac{Bp}{\widehat{Bp}} \text{ } \text{?} \\
 & \text{Forked} & \text{"Brown palp"} \\
 F_1 & \frac{\widehat{ff}}{Y} \frac{N}{\widehat{Bp}} \frac{N}{\widehat{Bp}} \text{ } \text{?} & \text{and} \quad \frac{Bp}{Y} \frac{N}{\widehat{Bp}} \frac{N}{\widehat{Bp}} \text{ } \text{?} \\
 & \text{Forked females} & \text{Non-Forked males}
 \end{array}$$

These Forked females were again crossed with "Brown Palp" males. In the  $F_2$  1/4th of the Forked females will have  $\frac{Bp}{Bp} \frac{Bp}{Bp}$  autosomes. Selecting for maximally dark palps, these autosomes will be selected. A "reconstituted" Brown Palp  $\widehat{XX}Y$  stock indistinguishable with regard to palp coloration from the pure line was derived after 4 generations. This result confirms the conclusion established by the examination of  $XO$  males. The presence of the  $Y$  in  $\widehat{XX}Y$  females does not suppress the character.

### RELATION OF SUPPRESSION TO THE $X/A$ BALANCE

It now seems clear that genes for Brown Palp situated on the autosomes can only be effective when the animal is a female. Exhibition depends on the same type of balance that determines female sexuality and depends on the equivalent of the hormone balance. The details of this could be elucidated by an examination of intersexes and gynandromorphs in a Brown Palp stock. It is not possible to obtain gynandromorphs in experimental numbers, but the production of intersexes ( $2X/3A$ ) is comparatively simple (Bridges 1921). Triploid females were crossed to males from the Brown Palp stock, and the offspring scored for Brown Palp. In their turn



the triploid female offspring were crossed to "Brown Palp" males and so on to the fifth generation. Owing to the inviability and infertility of the triploid Brown Palp females the experiment was brought to an end before a maximally Brown Palp triploid strain had been obtained. The figures (Table XVI) show that all the intersexes had the male palp coloration.

TABLE XVI

Generation of cross	Triploid females		Diploid females		Intersexes	
	Brown Palp	Non-Brown Palp	Brown Palp	Non-Brown Palp	Brown Palp	Non-Brown Palp
1	0	4	42	38	0	43
2	5	7	99	54	0	24
3	22	15	259	102	0	82
4	3	8	107	14	0	19
5	14	1	176	16	0	39

That Brown Palps are commoner among diploid females than among triploid females, especially in the earlier generations, is presumably due to the fact that the ratio of chromosomes from the Brown Palp stock to those from the original triploid stock is greater among the early diploids than among the early triploids. This difference of course declines as a triploid brown palp strain becomes established. It is well known that triploid intersexes have male sex-combs. Recent work on the localization of sex factors (Dobzhansky and Schultz 1934 and others) has led to the production of animals which have an  $X/A$  ratio between 0.66, the value for intersexes, and 1.00 for females. When the ratio nearly approaches 1 the sex-combs disappear and evidence of fertility in the now almost completely female type makes its appearance. In such studies on polyploid stocks the ratio of  $X$ -borne genes to genes carried by autosomes is considered without regard to their qualitative characteristics. In reality we have no reason for supposing that all the genes on the  $X$ -chromosome are relevant to the primary distinction between the sexes. It would be of considerable interest to find out whether the exhibition of Brown Palp depends upon the same genes as sex in general and, if so, at what stage in the disappearance of the sex-combs or the assumption of fertility Brown Palp also makes its appearance. Since fertility tests are somewhat laborious, it is possible that palp coloration might be used with advantage as an index of femaleness in studies on the localization of sex factors.

## DISCUSSION

We have already seen that differences of palp coloration between females are fairly widespread. Hence we may regard Brown Palp as an incipient secondary sexual characteristic of the species. Mr J. E. Collins of Newmarket informs me that palp coloration is a specific characteristic of some value in the classification of Diptera, and that it occasionally occurs as a secondary sexual difference characteristic of a particular species.

Brown Palp provides an example of an incipient secondary sexual characteristic without any known utility as a mark of sex recognition. Therefore we cannot as yet assume that it would be conserved by sexual preference. Should Brown Palp genes become irreversibly associated with high viability or fertility genes by means of an inversion, it could become established as a secondary sexual characteristic without the operation of sexual selection. It is not unlikely that a number of secondary sexual characteristics, such as that of the dogfish described by Brough (1937), became established in such a manner. If, on the other hand, palp coloration differences affect the mating preference of the male it might become a secondary sexual characteristic by a process of sexual selection. Probably the main agency in the evolution of the general physiological balance associated with sexual reproduction is the efficiency of the reproductive process itself. In so far as somatic differences are directly (or indirectly) an incidental by-product of the gene balance which determines the manifestation of the primary sex difference, the evolution of secondary sexual characteristics calls for no other explanation. Up to a point the conservation of sex-limited differences might even progress in spite of opposition arising from sexual preference. On the other hand, if a sexual preference in favour of assortative mating between dissimilar phenotypes arose at any stage in the emergence of a sex-limited character, it would greatly reinforce the type of natural selection already initiated.

In the evolution of secondary sexual characteristics depending on the type of gene balance demonstrated in the foregoing analysis of Brown Palp, it is highly unlikely that sexual preference would play any part at the beginning. On the other hand, it is not suggested that sex-limited differences can only arise in this way. In the introduction it has already been pointed out that there are two other possibilities, and we cannot therefore dismiss the sudden emergence of a clear-cut sex-limited character through the action of single dominant Y-borne genes, through the action of single dominant X-borne genes, or through non-disjunction and analogous phenomena. Should the character thus appear, sexual selection might be

the main agency by which it first became concentrated and was eventually established as a species characteristic. The relative importance of sexual selection and natural selection as means of establishing secondary sexual characters is therefore pre-determined by the genetic basis of a sex-limited character.

We are indebted to Professor J. B. S. Haldane, to Professor Lancelot Hogben, and to Dr H. J. Muller for criticism and advice. The investigation was completed with the aid of a grant from the Carnegie Trust for the Scottish Universities.

#### SUMMARY

1. By selection and inbreeding of *Drosophila melanogaster*, a sex-limited palp coloration difference confined to the female can be established. Palps of females belonging to such stocks are dark brown in contradistinction to the pale palps of the male. Both sexes transmit the genetic basis of the character.

2. In normal stocks of which the palp coloration of the female is relatively pale, there is a detectable difference between the colour of the female palp and that of the male. We may therefore regard palp coloration as an incipient secondary sexual characteristic throughout the species.

3. For the analysis of its genetic basis a phenotypic index based on arbitrary numerical symbols for different grades of intensity has been used in this investigation.

4. By a modification of the technique of "marked" chromosomes the contribution of individual chromosomes can be investigated. Brown Palp stocks have been crossed with *S/Cy*, *D/+* and with Oregon wild type.

5. The result of such matings shows that genes which contribute to the Brown Palp effect are not appreciably represented in the *X*-chromosomes of any of the stocks examined. They are to be found on both the second and the third chromosomes, and to be equally concentrated on the second chromosome of Brown Palp or other chromosomes.

6. The absence of the *Y*-chromosome in *XO* males did not result in the appearance of Brown Palp. The presence of a *Y*-chromosome in *XXY* females did not result in the suppression of the character. Hence the *Y*-chromosome does not contribute to the suppression of the character in the male.

7. Triploid stocks with autosomes predominantly from the Brown Palp stock were established. Triploid intersexes obtained ( $2X/3A$ ) were maximally pale, i.e. male in type. It is suggested that palp coloration might be used as an index of femaleness for the localization of sex factors.

8. The relative importance of Natural Selection and of Sexual Selection in establishing secondary sexual characteristics is discussed in relation to the genetic basis of Brown Palp and to other genetic types of sex-limited characters.

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## Spontaneous rhythmical impedance changes in the trout's egg

BY M. J. HUBBARD AND LORD ROTHSCHILD

*Communicated by J. Gray, F.R.S.*

#### INTRODUCTION

The phenomena recorded in this paper were discovered by accident. During experiments on the impedance of trout's eggs, it was found that under certain conditions the impedance varied in a rhythmical manner. This proved to be a property of live eggs and not of dead eggs and to be independent of the apparatus. The effect therefore was systematically examined.

Before discussing this effect, some analysis or definition of the electrical term impedance with respect to biological systems is desirable. The impedance  $Z$  of any system is its resistance to the passage of an alternating electrical current. If the current is direct, the impedance is replaced by the resistance which is related to the impressed electric current by the relationship

$$R = \frac{E}{I}, \quad (1)$$

where  $R$  = resistance,  $E$  = voltage, and  $I$  = current.

If, however, the source of electric current is alternating, e.g. of the form  $I = I_0 \sin \omega t$ , where  $\omega = 2\pi \times \nu$  (frequency) and  $I_0$  = the amplitude of the wave, the impedance of the system not only includes the resistance but also the capacitance  $C$ . (Inductances need not at present be considered in biological systems.) If the system consists of a resistance and a capacitance in series (see fig. 1*a*), the impedance is given by the expression

$$Z = \sqrt{\left(R^2 + \frac{1}{\omega^2 C^2}\right)}, \quad (2)$$

or, in complex notation,  $Z' = R + \frac{1}{j\omega C}$ , (3)

where  $j = \sqrt{-1}$ .

If the system consists of a resistance and a capacitance in parallel (see fig. 1*b*) the impedance is given by the expression

$$Z = \sqrt{\frac{R^2 + \omega^2 C^2 R^4}{(1 + \omega^2 C^2 R^2)^2}}, \quad (4)$$

or  $Z' = \frac{R}{1 + j\omega CR}$ . (5)

The impedance of a more complicated combination of resistances and capacitances (see fig. 7) can be expressed by a similar but more complicated relationship.

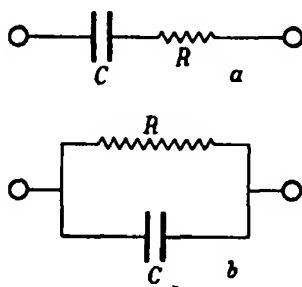


FIG. 1. *a*, Resistance and capacitance in series; *b*, resistance and capacitance in parallel.

In this paper we are concerned with changes of impedance in biological systems which do not consist of the wireless components shown in fig. 1. A change in resistance in such a system can be considered as a change in the ability of ions to pass through the protoplasmic surface; such a change could result from the partial occlusion or expansion of pores. A change in capacitance could be caused by the thickening or thinning of the protoplasmic membrane, as

$$C = \frac{DA}{4\pi r}, \quad (6)$$

where  $D$  = dielectric constant of the medium between the "plates" of the condenser,  $A$  = area of one of the plates, and  $r$  = distance apart of the plates.

From equation (6) it is evident that a change in dielectric constant will cause a change in capacitance equally well. A change in dielectric constant can be considered physically as a change in the ability of polar or oriented molecules to rotate according to the sense of the applied current. There are, of course, other equally good mechanical models.

### MATERIAL

Fertilized and unfertilized eggs of *Salmo irideus* Gibbons were used. Eggs were obtained by stripping ripe females in the laboratory. Sperm were obtained in the same way and tested for activity before use. Fertilization was effected by mixing dry eggs and dry sperm in a basin and, after about 1 min., pouring tap water over them. There are no immediate morphological signs that fertilization has occurred, but the four-cell stage is easily visible, particularly if the eggs are fixed in Bouin's fluid. If the four-cell stage has not been observed, it is usually necessary to wait from 4 to 5 days to be certain that the eggs have been fertilized. At this stage the embryo is clearly visible. If the fertilized eggs are kept in running tap water, it is comparatively easy to obtain about 80 % week-old embryos, provided that the female trout has not been kept too long unstripped.

Both fertilized and unfertilized eggs were kept in tap water for at least 12 hr. before being used in an experiment.

### APPARATUS

In principle, measurements were made with an alternating current bridge and detector. The egg was placed in an electrolytic cell in the unknown arm of the bridge (fig. 2). In practice a somewhat more complicated technique was used (fig. 3).

*Bridge.* The bridge was of the usual type, consisting of two equal non-inductive ratio arms  $a$  and  $b$ , the unknown arm  $c$  containing the egg shunted by a variable air condenser  $C_1$ , for balancing stray capacitances, and the standard arm  $d$ . The latter consisted of a variable non-inductive constantan resistance box  $R$  in parallel with a variable condenser box  $C_2$ .

*A.C. source.* A variable output heterodyne beat oscillator with low harmonic content was used. The frequency band was 0-50 kc.

*Detector.* Instead of the usual telephones, an electron oscillograph and resistance-capacitance-coupled amplifier with tuned filter output were

used, the bridge output being connected through the amplifier to the Y-plates of the oscillograph. The oscillograph was used in three different ways: (1) without using the X-plates (for moving film records), (2) with a conventional linear sweep circuit on the X-plates, and (3) with the X-plates in parallel with the transformed oscillator output through an

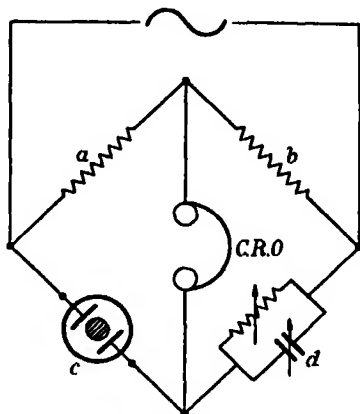


FIG. 2. Schematic A.C. bridge: *a* and *b*, ratio arms; *c*, unknown arm containing egg in electrolytic cell; *d*, standard arm; C.R.O. detector.

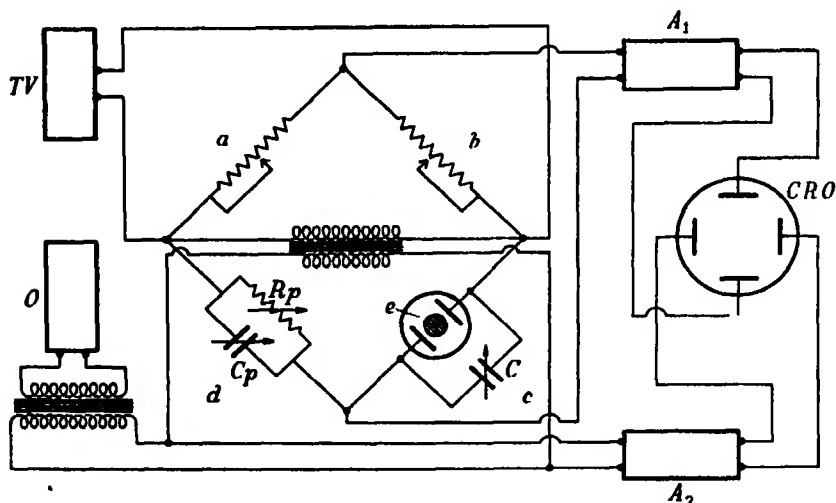


FIG. 3. *a* and *b*, ratio arms; *c*, unknown arm; *e*, trout egg in electrolytic cell; *C*, variable air condenser, max. 0.0008  $\mu$ F, in 0.000005  $\mu$ F steps; *d*, standard arm; *Cp*, variable air condenser, max. 1.0  $\mu$ F in 0.000002  $\mu$ F steps; *Rp*, variable resistance box, max. 11,111  $\Omega$  in 0.1  $\Omega$  steps; TV, thermionic voltmeter; O, oscillator; *A1*, amplifier; C.R.O. electron oscillograph; *A2*, amplifier.

independent resistance-capacitance-coupled amplifier. This latter method produces an ellipse or Lissajou figure on the oscillograph screen and is useful when very quick balancing is required, or for other special purposes (Cole and Curtis 1938).

*Cell.* Two types of cell were used (fig. 4*a*, *b*). Fig. 4*b* is a special cell used for one particular experiment; it is described later. Fig. 4*a* shows the type used for all other experiments. The electrodes were 1.0 cm. square and were platinized platinum. The egg was placed in the indentation in the middle of the cell. In all experiments 1.0 c.c. of tap water was placed in the cell.

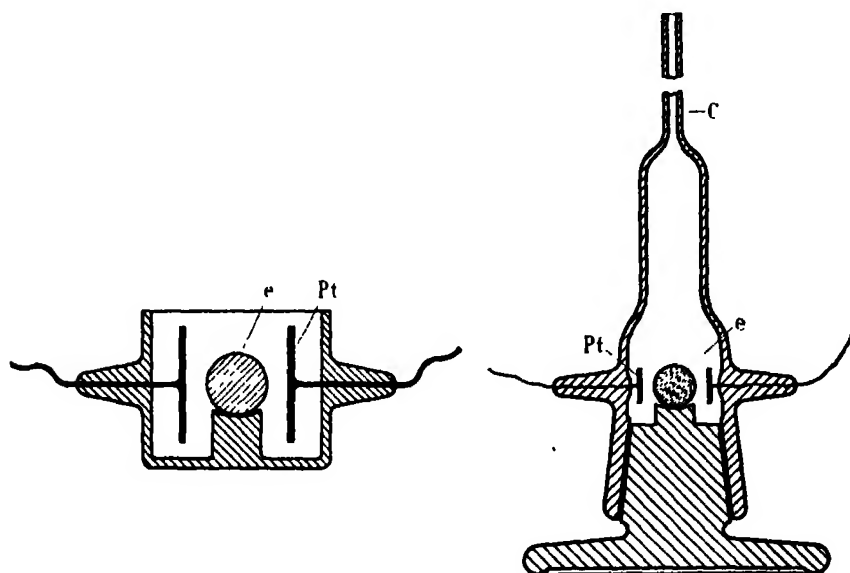


FIG. 4. Electrolytic cells. e, egg; Pt, platinum electrodes; C, capillary.

When the bridge is balanced, there is no potential across the bridge output and therefore no potential across the *Y*-plates of the oscillograph. If a moving film record is taken of the electron beam under these conditions (with no potential across the *X*-plates) and the film moves in the *X*-direction, a thin straight line will be recorded. If now the bridge becomes unbalanced due to an impedance change in the unknown arm of the bridge, a potential will develop across the *Y*-plates. This potential will be approximately sinusoidal as the system is energized by a sinusoidal source of e.m.f. and the amplitude of the beam deflection will be a function of the magnitude of the impedance change. If the camera speed is sufficiently fast to separate each cycle an ordinary sine wave whose amplitude will



vary with the magnitude of the impedance change will be recorded; if the camera speed is *not* sufficiently fast, each cycle fuses with the next and a black band will be recorded.

## RESULTS

*Unfertilized eggs.* Eggs were removed from a female and left in running tap water for 12 hr. One was then placed in the electrolytic cell, fig. 4*a*, and the bridge was quickly balanced by adjustment of the parallel resistance and capacitance in the standard arm of the bridge. A record of the excursion of the electron beam was then taken. Fig. 5 is a typical result. The frequency of the voltage applied to the bridge input is related to the camera speed in such a way that each cycle fuses with the next one. Fig. 6*a-e*, is a series of records each taken with the same egg but not the same as fig. 5, at different times. At the beginning of each record in fig. 6, the bridge was rebalanced.

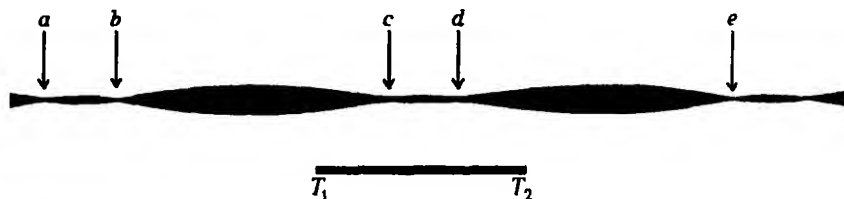


FIG. 5. 1 December 1938. Egg no. 19. Input frequency 5000~.  $T_1 - T_2 = 60$  sec.

In all cases there is a periodic change in impedance occurring with approximately constant frequency of 1.5 min. As this effect has not apparently been observed before, careful controls were performed to establish that it was not an artefact. Possible artefacts may be roughly classified into four groups:

- (1) Periodic changes in the apparatus.
- (2) Periodic convection currents in the tap water round the egg in its cell.
- (3) Periodic egg volume changes. This would be an artefact only in a special sense.
- (4) Only occasional eggs show the effect. This would also be a specialized artefact.

(1) is disproved as there is no effect when dead eggs are substituted for live eggs in the experiment. Similarly, if there is *no* egg in the cell, but only tap water, there is no effect. This and the evidence that (4) is false are shown in Table I.

When a trout's egg dies, the intracellular globulin, which is normally in solution, comes out of solution as a white precipitate (Gray 1920). The dead eggs used in these experiments were either ones which had died naturally as evidenced by globulin precipitation, or were obtained by placing live

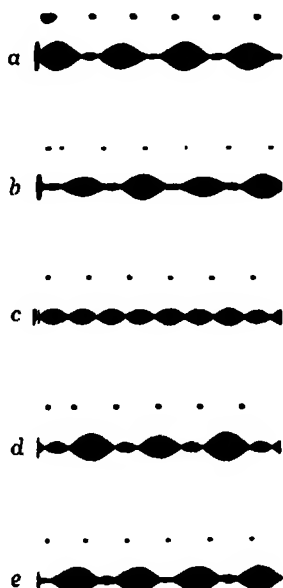


FIG. 6. 23 December 1938. Egg no. 94. Input frequency 4000  $\sim$ . Time-marker, minutes. See text for description of experiment.

TABLE I

Type of egg	No. of ex- periments	Periodic impedance change present
Live unfertilized eggs after 12 hr. in water	> 100	Yes
Unfertilized eggs killed by heat	25	No
Unfertilized eggs which died naturally	25	No
Tap water without eggs	10	No

eggs for 10 min. in tap water warmed to 80° C, when globulin again precipitates. Apart from these morphological changes, the dead eggs have very markedly different electrical characteristics, both as regards the e.m.f. across the egg-tap water interphase (Pumphrey 1931) and as regards the resistance and capacitance of the cell membrane (Rothschild, to be published).

The dead egg control is not entirely satisfactory as the impedance of a dead egg differs very little from that of the surrounding medium. A live

egg does not show any periodic impedance change when first put into tap water (see below), though its impedance is about the same as that of an egg which is undergoing the impedance cycle. This, in conjunction with the dead egg control, is good evidence that the impedance cycle is not an artefact.

Periodic convection currents in the tap water round the eggs are not responsible for the effect. Stirring the tap water by bubbling air through it does not abolish the impedance changes, nor does substituting paraffin oil for tap water.

Periodic changes in egg volume do not occur. This was established by photographing the eggs during the experiment and by means of the special electrolytic cell shown in fig. 4*b*. This is in essence a volumeter. The egg was placed in position, the base of the cell being detachable and the whole was filled with tap water, to about half-way up the fine capillary *C*. The meniscus of the water was observed through a horizontal microscope. The meniscus continually drifted owing to temperature changes. (This cell was technically very difficult to thermostat and a thermostated room was not available.) As, however, the drift due to temperature was slow and always in the same sense, any periodic volume change would have been easily detected. The smallest observable excursion of the meniscus corresponded to a 0.2 % change in egg volume. Similarly, no change in egg diameter could be observed photographically during an impedance cycle. The photographic technique could and in future will be made more sensitive.

The experiments mentioned above establish the fact that the effect is real, i.e. that unfertilized eggs which have been in tap water for 12 hr. show periodic impedance changes. Eggs do not show the effect when first put into tap water. It appears after about 7 hr. immersion and, in the unfertilized egg, it seems to continue until death, though after several days in tap water, the impedance changes become less regular and their period becomes longer.

The impedance changes appear to be spontaneous, though the exact significance of such a statement depends on a definition of the word spontaneous. This conclusion is based on the fact that the changes are entirely independent of frequency; reduction in oscillator output has no effect. The minimum convenient voltage across the cell with the apparatus previously described was about  $10 \times 10^{-3}$  V. This voltage may possibly be large enough to stimulate, though the amplitude of the impedance cycle is directly proportional to the oscillator output voltage. Furthermore, a cell which responds to a stimulating current of any frequency up to 50,000 cycles by an impedance change with a frequency of about 1.5 min. seems improbable.

Analysis and interpretation of these results may be divided into two parts; first, the analysis of the records in terms of impedance changes and second the analysis of the impedance changes in terms of changes in the resistance and capacitance of the vitelline membrane and/or possible changes in the resistance of the intracellular fluid or in the external medium.

The second part of the analysis is complicated even in relatively simple cases. In these experiments where the wave form of the impedance change may indicate that the resistance and capacitance changes comprising the impedance cycle may be out of phase, of different amplitudes and that the wave is not always sinusoidal, analysis is extremely complicated. The evaluation of specific resistances and capacitances from the observation of impedances at different frequencies has been developed in a series of papers by Cole (1928 *b*, 1937). The analysis is based on Maxwell's equation (1873) for the resistance of a homogeneous random suspension of spherically packed spheres and on Campbell (1911) and Cole's proof (1928 *a*) that the locus of the impedance vectors of a network consisting of two resistances and a capacitance plotted at various frequencies in the equivalent series form  $R - jx$  is a semicircle the position of whose centre depends on the dielectric losses in the capacitance in the network. Analysis of the trout's egg impedance cycle in terms of specific resistance and capacitance changes is too complicated and lengthy for inclusion in this paper, neither are these experiments entirely suitable for accurate analysis. It will be published elsewhere.

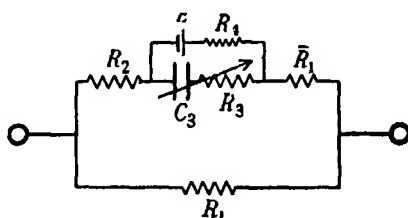


FIG. 7. An electrical network which is equivalent to the egg in the cell.  $R_1$ , resistance of suspending medium;  $\bar{R}_1$ , resistance of suspending medium between electrode and egg surface;  $R_2$ , resistance of intracellular fluid;  $R_3$ , resistance of cell membrane;  $E$ , e.m.f. across egg surface;  $C_3$  and  $R_3$ , polarization impedance of cell membrane. The impedance of the cell membrane cannot be represented as a pure resistance nor as a pure capacitance. Nor can it be represented as a combination of non-variable pure resistances and pure capacitances.

At a particular frequency any network consisting of any combination of resistances and capacitances is equivalent to either of the simple networks shown in fig. 1. This does not imply that the change in resistance or capacitance in the bridge standard arm necessary to balance a change in the unknown arm is numerically equal to the change in the unknown arm, for the egg is equivalent to a network of the type shown in fig. 7, where, apart from other differences, the capacitative element is of the polarization type; while balance is effected by a pure resistance and capacitance in parallel. The maximum and minimum values of the standard arm impedance [ $R/(1+j\omega CR)$  ohms] necessary to balance the egg during its impedance cycle maximum and minimum vary by app. 0.4 %. This percentage change is small, though it must be remembered that a null method is used in making measurements;

only the change round a mean value is recorded, the main egg impedance being balanced out. Though the percentage change in impedance is low, the percentage changes in standard arm capacitance and resistance are app. 4.0 and 0.4 respectively.

To deduce the actual type of impedance change which produces the record in fig. 5, it is advisable to consider the record in a slightly simplified form. Assume that the points *a*, *b*, *c*, *d* and *e* in fig. 5 represent bridge balance. (This is not the case as the lines at *b*, *c* and *d* are slightly thicker than at *a* and *e*, but this is discussed later.) One complete cycle lasting app. 1.5 min. then consists of an apparent large impedance change of long duration followed by an apparent small change of impedance of shorter duration. But from fig. 6, which shows a series of records taken on the same egg at different times (i.e. balanced on different parts of the impedance cycle) it will be seen that the relationship of these two phases can be varied according to the moment in time when the bridge is balanced; at a particular time of bridge balance, the two phases may even be of the same amplitude and duration (see fig. 6*c*). Consider hypothetically some periodic alteration in the structure of the protoplasmic membrane which produces an impedance change  $Z = Z_0 \sin \omega t$ . At any moment when the bridge is balanced, the impedance of the egg may be represented by a particular point on the sine wave. In fig. 8 different moments have been selected at random during the impedance cycle at each of which it is assumed that the bridge has been balanced. Thus at any time *t*, the standard arm impedance value necessary to balance the bridge (and thus the egg) is  $B_1$  in arbitrary impedance units (fig. 8*a*). This corresponds in practice to particular values of the parallel resistance and capacitance in the standard arm of the bridge. It will be remembered that *bridge* unbalance produces a bilaterally symmetrical oscillograph excursion, fig. 8, though this is obviously not the case with the function  $Z = Z_0 \sin \omega t$ . In the first case, fig. 8*a*, the bridge is balanced ( $B_1$ ) near the peak of the first half-cycle; therefore rebalance occurs in a short space of time, at  $B_2$ . The next balance occurs much later, as the wave does not return to the balance level  $B_3$  for more than the duration of a half-cycle.  $B_4$  occurs after a short time,  $B_5$  after a long period, etc. These periodic impedance changes are drawn at the side as bridge unbalances photographed on the oscillograph both with high and low frequency bridge input or carrier e.m.f.'s. In fig. 8*b* balance was effected nearer the median line of the wave, in *c* at the median line, in *d* and *e* at various distances below. In each case the relevant moving film record is drawn at the side. Although the frequency and form of the actual impedance change remains the same throughout, the form and apparent frequency of the recorded impedance changes vary according to the time

at which the bridge is balanced. The process of constructing the recorded impedance change from a known sinusoidal input impedance cycle (see above) can be reversed if the recorded impedance change is known. In fig. 9 the input impedance cycle has been reconstructed from the observed

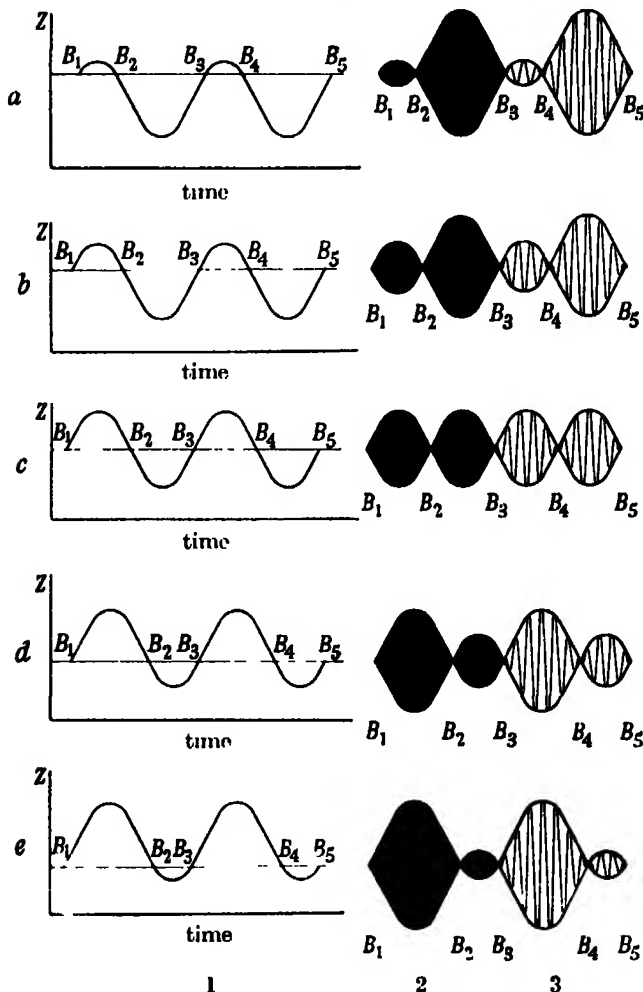


FIG. 8. 1. The function  $Z = Z_0 \sin \omega t$ . The horizontal line in each case represents bridge balance (ohms).  $B_1$  is the ohm-time co-ordinate at which balance is first effected.  $B_2, B_3, B_4$  and  $B_5$  represent successive bridge balances.

2. Detector record at high input frequency.  $B_1, B_2$  and  $B_3$  represent bridge balances.

3. Detector record at low input frequency showing separation of carrier wave.  $B_2, B_4$  and  $B_5$  represent bridge balances.

a1, 2 and 3; b1, 2 and 3 to e1, 2 and 3 show the effect of varying  $t$  in the co-ordinate  $Bt$ , first on the value of  $Z$  necessary to effect balance and secondly on the recorded change.

records in fig. 6. Comparison of figs. 6, 8 and 9 shows that the interpretation given above is consistent with the observed effects, with certain reservations discussed below.

This analysis is over-simplified. Fig. 10*a* is the same as fig. 6 and if this record is carefully examined it will be noticed that though there is a balance at *a*, there is a small residual unbalance at *b*, *c* and *d*. Sharp balance only re-occurs at *e*. This is not a property of the measuring apparatus. In some eggs this effect was very much more marked, e.g. fig. 10*b*. There is one

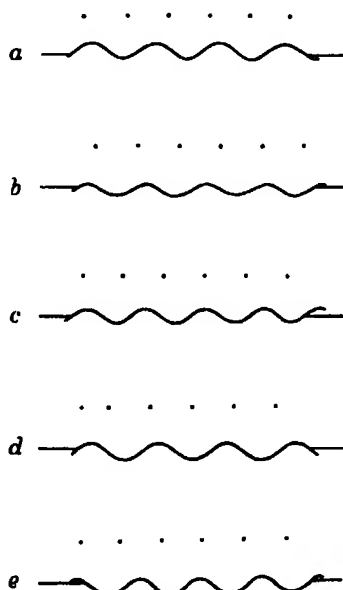


FIG. 9. Reconstruction of the input impedance cycle from recorded changes, fig. 6 *a*, *b*, *c*, *d*, *e*. Time-marker, minutes. The horizontal lines at the beginning and end of each record represent balance levels.

fairly simple explanation of this anomaly, though other more complicated explanations could probably be invented. The main impedance change is the one previously described, namely, a roughly sinusoidal variation in impedance, each cycle of which is recorded as two phases whose amplitude and duration vary according to the moment in time at which bridge balance is effected. Some change is, however, superimposed which only permits complete balance every fourth cycle. If the impedance cycle consists of two components, one of which is the change previously described and the other is a sinusoidal in-phase component of one quarter the frequency, the resultant change would be similar to the records in fig. 10. This is shown diagrammatically in fig. 11 which should be compared with

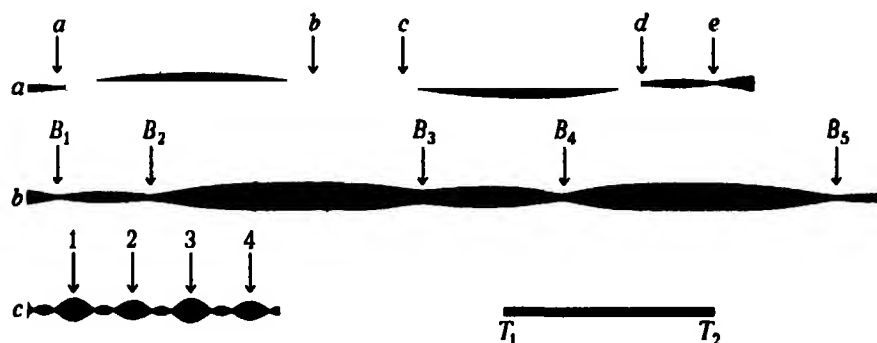


FIG. 10. (a) 1 December 1938. Egg. no. 19. Input frequency 5000 ~. Time,  $T_1 - T_2 = 60$  sec.  $a$  and  $e$ , balances;  $b$ ,  $c$  and  $d$ , partial balances.

(b) 5 December 1938. Egg no. 38. Input frequency 4000 ~. Time,  $T_1 - T_2 = 60$  sec.  $B_1$  and  $B_5$ , balances;  $B_2$ ,  $B_3$  and  $B_4$ , partial balances.  $B_3$  is more off-balance than  $B_2$  or  $B_4$ .

(c) 23 December 1938. Egg no. 94. Input frequency 4000 ~. Time; the arrows 1, 2, 3 and 4 are 1.8 min. apart. The amplitudes of phases 1 and 3 are greater than those of 2 and 4.

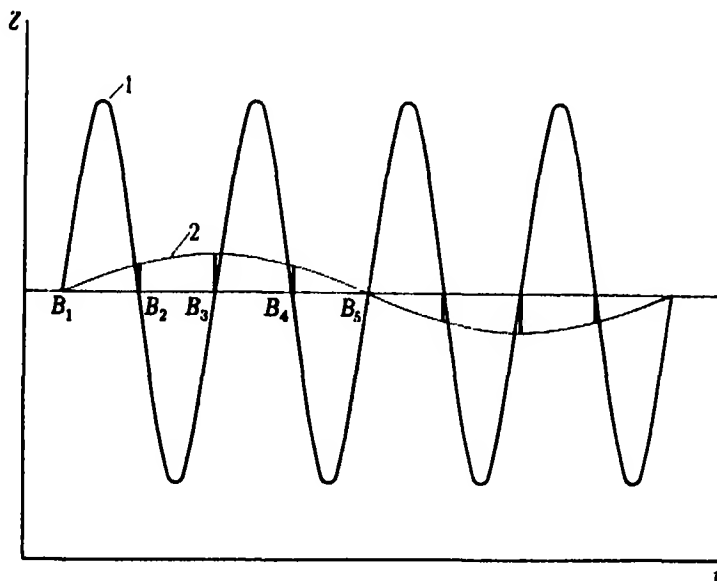


FIG. 11. The possible two components of the impedance cycle, (1) having 4 times the frequency of (2). A midway balance level has been selected as in fig. 6c. The off-balance at  $B_2$  is small owing to the small amplitude of the slow wave. At  $B_3$  the off-balance is greater than at  $B_4$  because the amplitude of the slow wave is at a maximum.  $B_4$  is the same as  $B_2$ , and  $B_5$  is the same as  $B_1$ , i.e. complete balance. The heavy vertical lines indicate the degree of off-balance at the various partial balance points.



figs. 10*a* and *b*, and fig. 12. It would be tempting to suggest that one component of the impedance cycle is due to a change in resistance while the other is due to a change in capacitance but this is not at present admissible. This analysis depends on the lower frequency component being of small amplitude compared with the other component. If the amplitude becomes significant, the degree of off-balance at partial balance should be observably greater at the end of the second cycle than at the end of the first; as after two cycles the amplitude of the lower frequency component is at a maximum. This is well illustrated in fig. 10 at  $B_3$ .



FIG. 12. 5 January 1939. Egg no. 138. *a-d*, effect of varying the time of bridge balance ( $Z_0, t$ ) on an egg which produced an impedance cycle which can be analysed into two components, one being four times the frequency of the other. Time-marker, minutes.

In unhealthy, unusually delicate, or stale eggs, the lower frequency component frequently had a relatively large amplitude. At the same time the impedance cycle was often of lower frequency in these eggs. A typical case is shown in fig. 12*a-d*. These records, which were taken on one egg which had been in tap water for 24 hr., illustrate very well the reduction in frequency mentioned above and the increase in amplitude of the lower frequency component which makes the off-balance at the end of the second cycle much greater than at the end of the first. These four records were all taken on the same egg, rebalance being effected between each record as in fig. 6. The effect of varying the moment of balance and thus the standard arm impedance necessary to effect balance is also well exemplified in these records; *a* and *d* happened to be about in the middle of the impedance cycle while *c* is so near the peak of the wave that there is some slight instability.

Throughout this paper it has been tacitly assumed that the impedance cycle is sinusoidal. This is not strictly the case and analysis is much complicated in these circumstances. Fig. 10c is an example where the simple harmonic analysis does not hold good. The resultant impedance cycle is approximately sinusoidal and there is the usual component with one-quarter the frequency of the main change. But this lower frequency component evidences itself as a periodic modulation, suggesting that it is not sinusoidal, though the main component is.

*Fertilized eggs.* The periodic impedance change is also present in the fertilized egg. It does not appear to differ in frequency, form, or time of inception from that observed in unfertilized eggs, but it does not continue indefinitely. At about the fifth day of development the effect ceases. The reason for this is not established, but it is possible that it may be correlated with the proliferation of cells round the vitelline membrane, which takes place about this time. This will be established by a more detailed morphological investigation. If the impedance cycle is a property of the vitelline membrane, it would be reasonable to expect that it would disappear when the vitelline membrane degenerates. The fact that the effect persists in fertilized eggs only for this period indicates that it is a property of the vitelline membrane rather than of that part of the unfertilized egg which develops into the embryo after fertilization.

## DISCUSSION

These experiments are as yet unfinished. The effect of changes in temperature,\* of varying the environment, the relationship between the impedance cycle and the death of the egg, the correlation between the morphology of the fertilized egg and the cessation of the effect and the effect of anaesthetics are obvious experiments to be carried out. These could not be done this year owing to the shortness of the breeding season.

There are few, if any observations on the trout's egg with which the impedance cycle can be correlated. Krogh and Ussing (1937) observed that the eggs absorbed water, presumably osmotically, for about 6 hr. after stripping, which is about the time when the impedance cycle begins, but there is no obvious way of relating these two phenomena. On general biological grounds, the frequency of the effect precludes comparison with other periodic changes of a similar nature. During nerve activity and probably during muscular activity, there is a change of impedance associated with the action

\* We have established qualitatively that an increase in temperature causes an increase in frequency, and vice versa.

potential. Spontaneous periodic potential changes occur in the brain and these may be associated with impedance changes, but there are few if any periodic changes which occur at such a low frequency.

An interesting problem which the impedance cycle raises is its relationship to fertilization. One of us (Rothschild 1935) has previously suggested that when an egg is laid, which in the uterus was in equilibrium with an environment of greatly different osmotic pressure from tap or river water, the sudden change in osmotic conditions in the environment may be an abortive parthenogenetic stimulus. In the frog's egg, for example, where one of the signs of activation is the appearance of the perivitelline space which permits the egg to rotate, rotation frequently occurs about 3 hr. after the unfertilized eggs have been put into tap water, though in fertilized eggs rotation occurs after 20 min. The existence of the impedance cycle in unfertilized trout eggs in tap water and in fertilized eggs may be an indication that trout eggs are partially parthenogenetically activated by suddenly being immersed in a medium of much lower osmotic pressure, though against this is the fact that both in fertilized and in unfertilized eggs the effect appears at the same time, i.e. about 7 hr. after immersion; also there is the possibility mentioned previously, that the impedance cycle is a property of the vitelline membrane rather than of that part of the egg which later develops into the embryo.

The significance of these results would be much simplified by an examination of other eggs to see if the effect is of general incidence, whether it is restricted to eggs of a particular type, or whether it is restricted to the trout's egg alone.

#### SUMMARY

1. Unfertilized and fertilized eggs of the rainbow trout show spontaneous periodic changes of impedance.
2. Measurements were made with an A.C. bridge and electron oscillograph as detector.
3. At an input frequency of 3000 cycles the maximum change in impedance per egg cycle corresponds to an app. 0.4 % impedance change in the equivalent parallel resistance and capacitance network in the standard arm of the bridge. The maximum capacitance and resistance changes in the latter are app. 4.0 and 0.4 % respectively.
4. The effect occurs only after about 7 hr. immersion in water.
5. The frequency is about 1.5 min.
6. Killing the egg abolishes the effect.

7. An increase in temperature increases the frequency and vice versa.
8. It is suggested that the impedance cycle consists of two components, (1) the main periodic change whose frequency is about 1.5 min., and (2) an in-phase periodic change of much smaller amplitude with a frequency of one-quarter that of the former.

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612.313.3:595.753

## Investigations of the mechanism of the transmission of plant viruses by insect vectors

### III. The insect's saliva

BY H. H. STOREY

*East African Agricultural Research Station, Amani,  
East Africa*

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[Plate 7]

For the type of virus transmission characteristic of leafhopper vectors, there is convincing evidence that the virus passes through the insect's body. The manner in which it emerges from the insect and comes to be inoculated into a plant is much less certainly known. It has generally been assumed that the saliva is the vehicle of the inoculation. For this assumption there is even now little direct evidence.

I now describe observations on the excretion of saliva by a leafhopper and attempts to demonstrate experimentally in this saliva the virus of which this insect is a specific vector.

#### MATERIALS AND METHODS

The material of these studies was pure "active" or "inactive" races of *Cicadulina mbila* Naude (Storey 1932*b*) and the streak-disease virus of maize. The general experimental methods and precautions against error were similar to those already described (Storey 1928, 1932*a*, 1933, 1938). Certain special methods will be mentioned in their appropriate sections.

#### OBSERVATIONS ON PUNCTURE AND SALIVARY EXCRETION

By the use of a small glass chamber similar to that described by Fife (1932), I have watched the stylets of *C. mbila* penetrating a wax membrane, 25–50 $\mu$  thick, and feeding on a 10% sucrose solution held behind the membrane. The chamber was placed on the microscope stage with the membrane vertical, and the stylets, puncturing across the field of view, were observed through a  $\times 20$  objective.

A moment after the insect's rostrum makes contact with the exterior of the membrane the maxillary stylets appear in the field of view (fig. 1, Plate 7). The mandibular stylets are rarely visible since they penetrate only a short distance and normally remain stationary once inserted, appearing to be wedged apart by the maxillary stylets, their serrated outer surfaces providing an anchorage in the membrane. The maxillary stylets advance smoothly into the fluid and retire jerkily, with rapid sliding movements of the two stylets one on the other. During the withdrawal, but not during the advance nor while the stylets are at rest, a colourless material flows from the tip of the stylets (fig. 2, Plate 7). This material neither visibly dissolves in the nutrient nor flows away, but sets to a gel (fig. 3, Plate 7). Repeatedly the maxillary stylets advance through the coagulating mass and retire with further ejections, moulding it internally to the form of a sheath, either simple (fig. 7, Plate 7) or branched (fig. 9, Plate 7). The sheath material sets so firmly that on withdrawal, even within a few seconds after puncture, a tubular cast of the stylets remains, sometimes, if the wax membrane be thin, showing the clear cut serrations of the mandibles. I have even observed air to enter the sheath following a sudden withdrawal of the stylets (fig. 7, Plate 7).

Although occasionally a hungry insect may start to feed at once, its stylets remaining stationary after the first movement of penetration, the

usual course is for it to build a substantial sheath by repeated to-and-fro movements during the first minute. At irregular intervals thereafter additions to the sheath may be made by further movements. If suitable particles be suspended in the nutrient fluid, their movements reveal a flow of the fluid into the stylet-tip, both during forward movements and while the stylets are at rest (figs. 4 and 6, Plate 7). This flow may continue uninterrupted for a few minutes or for an hour or more. Between periods of sucking the insect rests with its stylets partly withdrawn down the sheath, the end of which is previously sealed by the ejection of a further quantity of sheath material (fig. 5, Plate 7). In nutrient fluids buffered to hydrogen-ion concentrations of approximately pH 3, 5, 7, 8 and 10, the process of sheath-formation appeared to take the same course and in all a firm sheath was formed. I observed only one marked deviation from the normal procedure, which occurred in both acid and alkaline ranges of the nutrient. Owing to the first droplet of the sheath material becoming detached from the inner surface of the membrane and being without anchorage, it was carried forward on the advancing stylets, and by accretion of more material solid strings were built up two to three times as long as the stylets (fig. 8, Plate 7). This abnormality is clearly a result of the artificial conditions and does not differ in essentials from the usual sheath-making.

It can hardly be doubted that the sheath material is a product of the salivary glands. It is less certain however that this material is their sole product. I have devoted much care to the search for evidence of a flow from the stylets of any other form of saliva. In this search I have relied primarily upon the assumption that any fluid not homogeneous with the nutrient fluid would, upon issuing from the stylets, produce striae or some similar visible disturbance within the nutrient fluid. I have confirmed that such a flow from a capillary glass pipette can be readily seen, particularly under the oblique illumination obtained by a sideways displacement of the substage diaphragm of the microscope. If 10% sucrose be injected into distilled water, the issuing fluid is seen as an unevenly illuminated cone; as the rate of flow falls the size of the cone decreases, until only a small bright spot remains visible at the tip of the pipette; finally this spot disappears, even though a very slow flow—as shown by particles present in the ejected fluid—continues. If 10% sucrose be injected into 9% sucrose the effect is still visible with a strong flow, but as the rate of flow falls the bright spot disappears at an earlier stage. If the two fluids are homogeneous the flow is never visible.

Under similar conditions—as well as by direct and dark-field illumina-

tion—I have watched the stylets of *C. mbila* while feeding upon 1 and 20% sucrose solutions. On no occasion have I certainly seen a bright spot at the stylet-tip in either fluid. Particles in the nutrient fluid drawn to the stylet-tip by the insect's suction may pulsate regularly, doubtless in relation to the action of the pharyngeal pump. Very occasionally these particles may be shot away some distance and be drawn back immediately to the stylet-tip. This movement certainly denotes a momentary outward flow. Usually it has occurred without any bright flash; but on rare occasions there has been a suggestion of a flash, so indefinite, however, that I hesitate to trust the observation. Sometimes minute droplets, apparently of the gelling material, have appeared at these times and have become entangled with the particles drawn to the stylet-tip. Although I feel very uncertain about the interpretation of these phenomena observed only infrequently, I suggest that the fluid ejected was homogeneous with the nutrient, and was in fact an outward flow down the suction canal of the stylets caused by a momentary failure of the valve mechanism. This outward flow was observed only when a mass of particles had collected on the stylet-tip and may have been a voluntary action intended to clear the choked canal.

While the insect is sucking, particles in the nutrient fluid may be seen to accelerate to the stylet-tip. Some remain there (fig. 4, Plate 7); others approach, but, after striking the stylets or stopping before reaching them, move away again with a corresponding deceleration. This observation suggested that an outward flow was occurring simultaneously with the inward one. I believe, however, that it is due solely to eddy currents produced by the suction; for exactly similar movements of approach and recession were exhibited by particles in the neighbourhood of a micro-pipette into which a steady flow of the fluid was being drawn.

Fife (1932) has reported that the fluid exposed to the feeding of *Eutettix tenellus* Baker becomes rapidly alkaline, and I have confirmed this for *Cicadulina mbila*. It must be concluded that an electrolyte issues from the insect into the fluid, either as a flow independent of the sheath-forming saliva or as a soluble component of this saliva at the time of its ejection. The following observation appears to support the second explanation. On one occasion an insect punctured the membrane close to the glass bottom of the observation chamber, upon which had collected a uniform layer of Indian ink particles. Immediately after the first ejection of sheath material the particles were swept away, leaving a clear zone extending some distance around the sheath (fig. 6, Plate 7). It did not appear that this phenomenon was caused by any gross movement of the fluid, but rather

by a momentary change in the surface tension at the interface between the nutrient and the glass. I concluded that a soluble component had diffused from the salivary mass at the moment of its ejection.

There can be no doubt that the stylet-sheath seen in process of formation in a fluid is identical with that found in histological preparations of the leaf (Bennett 1934; Storey 1938). Nor can there be any doubt that this sheath is wholly of insect origin, as F. F. Smith (1933) has concluded from microchemical studies. As I have reported (Storey 1938), the sheath in the leaf shows two layers with different staining reactions; so also does an isolated sheath formed in a sucrose solution. Histological preparations suggest that the process of sheath formation in the leaf is similar to that in a fluid, although probably less rapidly completed (Storey 1938). There are indications that once the phloem is entered the stylets are not again moved to another tissue. The saliva, being ejected into a confined space in the leaf, may flow along elongated cells before gelling (Storey 1938, fig. 4, Plate 28). Bundle sheath and mesophyll cells receiving saliva have degenerated cell contents, but adjacent cells remain apparently unaffected. *Cicadulina* differs markedly in this respect from some jassids that have been studied (K. M. Smith 1926; F. F. Smith and Poos 1931).

#### VIRUS IN THE SALIVARY GLANDS

I have carried out experiments in which organs taken from the bodies of infective "active" *C. mbila* have been inoculated into the abdomens of non-infective ones. There is reason to expect that these insects would prove to be infective in a subsequent test, if the organ inoculated contained any considerable amount of virus\* (Storey 1933).

The technique employed was to dissect the organ required from an anaesthetized insect under sterile 0.7% saline; to pass this organ successively through several drops of sterile saline, each transfer being made with a fresh sterile needle, or with a micro-pipette, if the organ could not readily be picked up by needle; and finally to inoculate the organ by one of the following methods. (1) Portions of the organ were implanted by needle into the abdomens of the insects receiving the inoculation. This method was not very satisfactory since, however rapidly the transfer was performed, the inoculum usually dried on the needle before it could be

\* It is impossible at present to express this more exactly. There are indications however that the minimum quantity of virus that can be revealed by this inoculation test may be relatively large; and that the test is less sensitive than that by feeding the suspected virus-containing material to insects.



inserted. (2) Portions of the organ were drawn up into a micro-pipette, of a bore just large enough to allow them to enter, and injected together with excess saline. (3) The organ was crushed in a minute droplet of saline and the suspension taken up by micro-pipette and injected. In the majority of experiments parts of the organ from each individual infective insect were inoculated into several non-infective ones; if any of these became infective the result was recorded as a single positive.

The organ with which this paper is particularly concerned is the salivary gland. The results of experiments in inoculating this gland appear in Table I. In each experiment one or two of the inoculated insects became infective.

TABLE I. THE INOCULATION OF PORTIONS OF SALIVARY GLANDS, DISSECTED FROM INFECTIVE "ACTIVE" *CICADULINA MHILA*, RECENTLY FED ON VIRUS, INTO NON-INFECTIVE "ACTIVE" INSECTS

Year and exp.	Treatment of dissected glands	Method of inoculation	No. of glands inoculated	
			Total*	Positive
1933—R 192	Washed successively in 5 drops saline	Needle	9	1
1934—R 195	Washed in 1 drop saline	„	4	1
1938—R 216	Washed successively in 2 drops saline	Pipette	11	1
1938—R 224	Washed successively in 3 drops saline	„	9	2
1938—R 230	Washed successively in 3 drops saline	„	9	2

\* Includes only those experimental inoculations in which one or more of the inoculated insects survived more than 2 days during the test for their power to cause infection.

The interpretation of these results is by no means certain. The fact, already reported (Storey 1933), that virus may be present in the blood, which bathes all the organs of the body, raises a doubt whether the few positive results were not due to virus carried in blood adhering to the gland and not removed by the washing given. On the other hand, it is possible that, in the process of washing, virus present in the gland became largely inactivated; I observed that the gland, translucent at first, rapidly became opaque in saline. I have tried to obtain a basis for judgment of these possibilities by experiments with other organs dissected from infective "active" insects, all fed on diseased plants until removed for experiment.

(1) *Ovarioles*. Dissected from 11 adult insects, washed in 2 drops saline; portions of each inoculated by pipette into 2 non-infective insects. Results—all negative.

(2) *White (posterior dorsal) mycetome*. Dissected from 11 adults, washed in 3 drops saline (transferred by pipette), inoculated by pipette. Results—all negative.

(3) *Yellow (anterior ventral) mycetome*. Dissected from 20 nymphs, washed in 3 drops saline, fragments inoculated by pipette. Results—all negative.

(4) *Malpighian tubules*. Dissected from 9 adults, washed in 3 drops saline, inoculated by pipette. Results—all negative.

(5) *Mid-intestine*. (a) Dissected from 12 adults, washed in 5 drops saline, inoculated by needle. Results—8 positive.

(b) Dissected from 11 adults, washed in 2 drops saline, inoculated by pipette. Results—11 positive.

(c) Dissected from 10 adults, washed in 2 drops saline, inoculated by pipette. Results—10 positive.

Consistently negative results were thus obtained in Exps. 1-4 with certain organs, that were as likely to carry a surface contamination of blood as the salivary glands. Washing in two or more drops of saline appears therefore to free them effectively from this contamination. The intestine on the other hand was likely to contain virus, since the insects had recently fed on a diseased plant. Passing the intestine through five drops of saline did not inactivate the contained virus; both the needle and pipette methods of inoculation were successful.

On the basis of this evidence, I conclude that the few positive results from inoculation of salivary glands were not due to technical error. I also conclude that the large proportion of negative results was not due to simple failures of the technique. It is still possible that post-mortem changes in the glands may have inactivated the greater part of the virus contained in them, in spite of the evidence that post-mortem changes in the intestine do not inactivate contained virus. On the most likely interpretation, virus is present in the salivary glands of *C. mbila*, either on infrequent occasions or in such dilution that my methods only rarely revealed it.

#### VIRUS IN A FLUID FED ON BY INFECTIVE INSECTS

I have performed many experiments designed to demonstrate virus in fluids upon which infective *C. mbila* had fed. The fluids used were: (1) healthy maize juice, with 10% sucrose added; (2) distilled water with

10% sucrose; (3) same as (2), with 0.5% pepsin or trypsin added, the reaction being adjusted to a pH favourable to the enzyme used; (4) same as (2) with Indian ink added. Some added sugar was necessary in order to make the insects feed freely on any fluid. Carter (1928), in somewhat similar studies on *Eutettix tenellus*, used fluids compounded with a variety of sugars; but in his work none seemed to be better than a simple sucrose solution. In my experiments, the proteolytic enzymes were sometimes added in the hope that the salivary sheath (reported to be largely protein (F. F. Smith 1933) might be dissolved and that virus, if held in the gel, might be released. Indian ink was incorporated, since observations, reported above, suggested that an outward flow might occur from the stylets when the canal entrance was choked by suspended particles.

The first method of testing for virus was to inoculate the fluid by needle into the abdomens of non-infective "active" *Cicadulina mbila*. The fluid (in one experiment 2 c.c.; in all others only a thin film) was held behind a membrane of "Baudruche capping skin", and about 50 infective insects were allowed to feed on it for one day. Upon removal of these insects, the fluid was at once used for inoculation. In six separate experiments a total of 170 non-infective insects were inoculated with it. In addition, 31 insects received material scraped from the inner surface of the membrane. In two further experiments a wax membrane was substituted, and portions of this, known to be carrying many salivary sheaths, were implanted into the abdomens of 60 insects. In another the feeding medium was solidified with gelatine and scrapings from its surface immediately below the membrane were inoculated into 33 insects. None of the 294 inoculated insects became infective.

A second method of experiment was to allow non-infective insects to feed on the fluid into which virus was presumed to have been inoculated by infective ones. In four experiments 10-40 infective insects fed for 1-3 days on 2 c.c. of fluid; thereupon non-infective insects fed for one day through the same membrane. A total of 44 non-infective insects all remained non-infective. Carter (1928) reported some successful experiments on these lines; his results will be discussed later.

In further experiments the volume of fluid was reduced to a film held between two membranes. On one side of this double membrane 50-150 infective insects were caged; on the other, usually 50 non-infective insects. All the insects fed for 1-2 days simultaneously. Thereupon the originally non-infective insects were tested, in groups of 10 or as a single group, on a succession of maize plants, these tests extending over at least 10 days. Of sixteen experiments using this technique fourteen gave negative results;

in these, all of 690 non-infective insects remained non-infective. In one successful experiment, two groups of 10 insects out of three caused infection of the test plants; in the other, a single group of 50 insects did so. The fluids used were healthy maize juice with sucrose in one experiment and distilled water with sucrose and pepsin in the other. It is unlikely that the constitution of the fluid was important, since both kinds were used also in experiments that gave negative results.

A point of great interest arises from the behaviour of these successful groups in later tests (fig. 10). No group caused infection in any test but the first. Owing however to the death of some members of each group during the first test, the evidence is not conclusive that members of the groups that were infective during the first test had ceased to be so during the second.

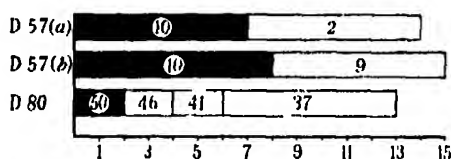


FIG. 10. Tests of insect-groups from "double-membrane" experiments. The history of each group is to be read horizontally; each rectangle represents a test, in which the insects fed on a single maize plant for the number of days shown by the length of the rectangle. If black, the test was positive for streak infection; if blank, negative. The figures in each rectangle are the numbers of insects entering each test; differences are due to deaths during the preceding test.

Five experiments were performed to test an early hypothesis that the success in the preceding experiments was due to transmission of virus as a simple contaminant of the stylets. In each, 100 infective insects fed through a membrane on a film of fluid for 3-24 hr.; the infective insects were then replaced by 50 non-infective ones and the membrane with its adherent film pressed into contact with the leaf of a maize plant, and so held for 2 days. It is known that *C. mbila* can puncture through a membrane into an underlying leaf, and, if infective, can successfully inoculate that leaf (Storey 1938). In the present experiments the stylets passed through a film of fluid, presumed to contain inoculated virus, directly into the plant. No test plant, however, became diseased; nor did any when the same insects were transferred to other plants by the usual technique.

#### VIRUS IN A LEAF FED ON BY INFECTIVE INSECTS

When infective *C. mbila* feed on a leaf of a maize plant, they inoculate virus into it, for the plant becomes diseased. It is reasonable to assume

that they inoculate virus also into a detached piece of leaf. I have attempted, by using methods similar to those of the preceding section, to demonstrate experimentally that this virus is present in the inoculated leaf at about the time of inoculation or shortly afterwards.

Five experiments were performed, in each of which 50–100 infective insects fed for 3–96 hr. on detached pieces of leaf, or on the tip of an attached leaf. The leaves were then crushed and their juice inoculated into non-infective insects. In all, 175 insects were inoculated, but none became infective. Bennett (1935) has devised a method of obtaining the sugar-beet curly-top virus from expressed juice by precipitation with alcohol. This method is effective also with the maize streak virus if applied to the juice of fully diseased leaves. The precipitate obtained from a leaf exposed for one day to 250 infective insects failed, however, to make infective any of 32 insects that received it by inoculation. Part of the precipitate was taken up in 10% sucrose solution and fed through a membrane to 30 non-infective insects. Again none became infective.

In a series of experiments non-infective "active" insects fed on a leaf simultaneously with infective ones. By one method 50 non-infective nymphs were caged for 2 days with 50 infective adults on detached pieces of leaves. In the following tests all the nymphs were proved to be still non-infective. This experiment was repeated with 100 insects of each category; the results were again negative. A second method was to set up the leaf to form a septum between two cages containing infective and non-infective insects respectively. Approximately 5 sq. cm. of leaf was exposed for 1–2 days to 50–100 insects on each side. I have shown that *C. mbila* can penetrate to the phloem from either surface (Storey 1938); since the insects were closely crowded on this small area of leaf, it is likely that individuals from each category often punctured the same vascular bundle. At the end of the feeding period the non-infective insects were usually separated at random into groups of 10 and given a succession of tests. I have carried out eleven independent experiments on these lines, of which six gave negative results. In these six a total of 380 insects all failed to become infective. In five experiments a small number of infections were recorded, as detailed in Table II. The successes all occurred with detached leaves standing in water; the failures with both detached and attached leaves.

If we accept the truth of the independence hypothesis (Storey 1938), we can calculate, in the manner given by Fisher (1934, p. 63), the approximate percentage of infective insects obtained in each experiment. The percentages appear in the last column of Table II. Although on such small

samples these figures are subject to considerable error, they give a true indication how small is the proportion of the insects that became infective—in even the most successful experiment no more than 5%.

TABLE II. DETAILS OF FIVE "LEAF-SEPTUM" EXPERIMENTS, IN WHICH 50-100 INFECTIVE *CICADULINA MHILI* FED FOR ONE DAY FROM ONE SIDE OF A MAIZE LEAF, AND NON-INFECTIVE ONES FROM THE OTHER. SIX SIMILAR EXPERIMENTS GAVE ONLY NEGATIVE RESULTS

Year and exp.	Tests of originally non-infective insects			Calculated approximate percentage of infective individuals
	No. of insects in each group	No. of groups caused infection	No. of groups not infective	
1933—U 3	10	2	6	3
1933—U 4	10	1	12	1
1938—U 36	10	4	6	5
1938—U 38	10	1	9	1
1938—U 40	10	1	9	1

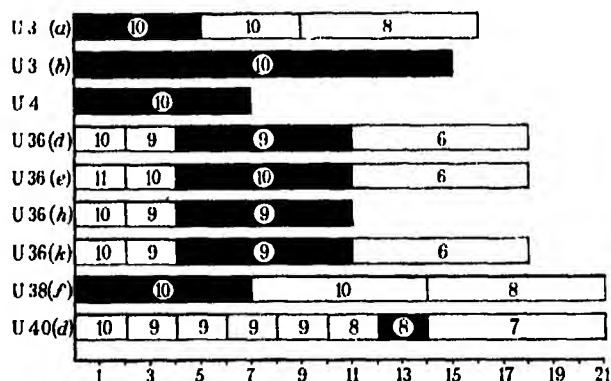


FIG. 11. Tests of insect groups from "leaf-septum" experiments.  
See explanation under fig. 10.

As in the "double-membrane" experiments already recorded, the successful groups of insects never caused infection in more than one of a series of tests (fig. 11). Four groups caused infection in the first test; of these, two groups were carried to later tests, which were invariably negative. In each of these groups all ten members survived to the second test; the insects that were infective during the first tests were therefore no longer so during the second. Five groups failed to cause infection during early tests but succeeded once each in a later test; thus the group in U 40 failed in six 2-day tests, succeeded in the seventh, but failed again in the eighth.

In order to test the hypothesis that the few positive results were attributable to contamination of the insects' stylets, the "leaf-septum" experiment was repeated with an "inactive" race of *C. mbila*. "Inactive" insects should be as efficient in this method of transmission as "active" ones. Seven experiments, however, involving the testing of 580 "inactive" insects, gave only negative results.

The preceding evidence appears to conflict with that of certain experiments reported in an earlier paper (Storey 1928). Actually the conflict is not direct, since these experiments were differently designed. In two experiments, a total of 9 out of 58 *C. mbila* became infective by feeding on the inoculated leaf for periods of from one to several weeks. In a second series, non-infective insects were caged on the lower part of a leaf during the time that it was being inoculated in its tip. One experiment was negative, while two experiments, in which the insects fed for 8-14 days, gave a total of 15 infective insects out of 68. A difference between these experiments and the ones reported in the preceding paragraphs is that here the insects continued to feed for several days after symptoms had developed fully on young leaves of the plants. The virus had by this time undoubtedly multiplied greatly in the plant, and conceivably had reinvaded in quantity the leaves through which it was originally inoculated. I doubt, however, whether this can be true, for I have shown that the green parts of a fully diseased plant appear to contain no virus that can be taken up by *C. mbila* (Storey 1928, 1938).

Another disturbing point is that I have almost completely failed to confirm the earlier results. Three experiments in feeding non-infective insects for several weeks on the inoculated leaf gave only a single infective individual out of 153. Four experiments in feeding the insects on the lower part of the inoculated leaf were all negative. I have no reason to doubt the soundness of the earlier experiments, although since they were done my methods have been improved and, if a choice must be made, the later results must stand as the more reliable. It is possible that the earlier positive results are strictly comparable with the few positives obtained in the "leaf-septum" experiments here reported; if so, some unknown difference in the conditions of experimentation must account for the greater relative success of the earlier ones. Unfortunately the infective insects in the earlier experiments were not given a succession of tests which would have shown whether they also lost infective ability within a few days.

## THE MECHANICAL INOCULATION OF PLANTS

The streak virus is one of a large class of viruses that have resisted all attempts at transfer from plant to plant by mechanical inoculation. Employing all the usual methods I have inoculated expressed juice of diseased maize plants, known to contain virus (Storey 1932*a*), into over 600 plants in 43 separate experiments, invariably with negative results.

I have also inoculated plants with fluids obtained from insects either known or believed to contain virus: 180 with the general body contents of infective insects; 30 with salivary glands dissected from infective insects; 210 with a variety of fluids previously fed to infective insects; 80 with the juice of pieces of healthy maize leaf after exposure to the feeding of many infective insects. Again all experiments were negative.

A needle is a crude inoculating instrument compared with the insect's stylets. I have tried to employ the stylets of living *C. mbila* as the inoculating instrument, by causing them to become contaminated with virus during their passage into the leaf. Certain experiments on these lines have already been described. I have injected virus-containing leaf-juice into the intercellular spaces of a leaf, and then caged non-infective insects on the injected area. When these insects were of an "inactive" race, no infections resulted in 40 trials. When they were non-infective "active" insects and were allowed to feed no longer than 2-3 days, again 40 trials were all negative. Four of these 40 insects, however, proved to be infective in a second test. When, in a similar experiment, the "active" insects continued to feed for 12 days on the injected plants, one plant out of 20 became diseased. Evidently therefore the insects at least occasionally penetrated the injected tissue and sucked in some of the fluid that had been injected; in consequence they became infective after the lapse of a latent period of more than 2-3 days and less than 12 days (Storey 1928). Although for this reason we may infer that the insects' stylets became contaminated with virus during the course of their penetration of the leaf, nevertheless they failed to infect any plant as a result.

## DISCUSSION

A surprising feature of this work has been the difficulty in demonstrating virus in a leaf inoculated by infective *C. mbila*. There is reason for thinking that the virus does not immediately multiply in the plant at the point of its inoculation (Bennett 1934; Storey 1938); but even the virus constituting the very many infective doses known to have been inoculated



can but rarely be demonstrated experimentally. I have considered two possible hypotheses that would explain this difficulty. The first is that the streak virus undergoes a transformation during passage through *C. mbila*; that it issues from the insect in a form that is capable of infecting a plant, but incapable of making another insect infective. On this hypothesis the few positive results were due to transfer of the new form of virus mechanically on the surface of the stylets.

The idea that a virus can be transmitted as a contaminant of the stylets has recently come to be widely accepted as explaining the phenomena of transmission encountered with the majority of aphid vectors (Doolittle and Walker 1928; Hoggan 1933). Watson's recent work (1936, 1938), however, questions its truth even for this type of transmission, which differs in many features from that considered in this paper. The evidence here presented shows that *C. mbila* fails to inoculate successfully the normal form of the streak virus by contamination of its stylets, even when this contamination occurs during the passage of the stylets through the mesophyll on their way to the phloem. It fails also to inoculate in this way the hypothetical second form of the virus, when similar precautions are taken to ensure the maximum opportunity for contamination.

The hypothesis of a virus transformation relied mainly on the early findings that the few experimental infections obtained were always in the first of a succession of tests. Virus might be carried mechanically into the first of the test plants, but it is highly improbable that any would remain on the stylets to be carried into the second. In certain recent experiments infections appeared in tests as late as the third and seventh. Furthermore, as mechanical carriers "inactive" *C. mbila* should be as efficient as "active" ones; but in fact they always failed. On this evidence, the hypothesis must be abandoned.

My second hypothesis, which I believe to be true, is that the infrequency of positive results was due to the extreme dilution of the virus presented to the non-infective insects in all my experiments. This implies that a dose of virus sufficient to infect a plant may be very small, and that in fact the infective insect emits virus in only very small amounts. Although a leaf might have received many infective doses, yet the total quantity of virus received might be so small that only rarely in my experiments did enough reach a non-infective insect to make it infective.

This hypothesis receives support from the evidence that even in the few successful experiments the insects possessed a very low infective ability. Freitag (1936) has shown for *Eutettix tenellus* that the probability of infection in any one of a succession of tests and the rate of fall of this

probability with time depend on the original quantity of virus taken in by the insect. I have found that with *Cicadulina mbila* also the original dose of virus is one of the factors determining infective ability. The ability of the insects, in the experiments here described, to infect only once in a series of tests is that which would be expected if they had taken up only a small amount of virus.

Turning now to the experiments in feeding insects on fluids, we see that positive results followed only when precautions were taken to reduce to a minimum the dilution of the virus presumed to have been inoculated. Again successes were few, and the successful insects showed a low infective ability. Carter (1928) was able to obtain infective *Eutettix tenellus* by feeding them on apparently large volumes of solutions on which infective insects had previously fed. His experiments are incompletely reported; but it is evident that a considerable proportion failed entirely, while it appears that in the successful ones relatively few insects became infective. In only one experiment is a second test of the insects reported; an unstated number caused infection of three plants out of four in the first 24 hr. test and of one only out of four in the second. Although Carter's technique of successive membrane-feeding failed with *Cicadulina mbila*, his results appear not to differ fundamentally from my own.

The view has been widely held that the ability of certain vectors to remain infective for long periods after a single original virus feed implies that the virus multiplies in the insect's body. If now my hypothesis of an extreme economy in the insect's output of virus be accepted, the argument for multiplication is greatly weakened. The multiplication hypothesis has been recently disputed on other grounds (Freitag 1936; Bennett and Wallace 1938).

The problem how *C. mbila* inoculates the virus remains still unsolved. I believe that my evidence is reliable that virus can be present in the salivary glands, although it is uncertain whether it is present in such small quantities as to be detectable only with difficulty, or is present only on occasions, presumably when the insect is in some particular physiological state. Bennett and Wallace (1938) also reached the tentative conclusion that virus could be present in the salivary glands of *Eutettix tenellus*, but only in small quantities. I agree with them that the salivary glands do not act as a reservoir of virus. But the fact that virus can be detected in these glands, whereas in *Cicadulina mbila* it cannot be detected in other organs (except the intestine), favours the hypothesis that the insect's saliva carries the virus into the plant.

The number of positive results obtained in the "leaf-septum" experi-

ments was not of a different order of magnitude from that in the "double-membrane" experiments. There is consequently no reason for thinking that the insect withholds virus in some manner while feeding on a fluid medium. The observations that I have made on insects feeding through a membrane are therefore relevant to this problem; while under observation the insects must have emitted virus. The only material known to have been emitted is the saliva that gels to form the sheath. The emission of this material is essentially a part of the initiation of puncture; when the insect settles down to suck food, no more of it is ejected. And yet I have shown (1938) that punctures maintained for less than 5 min. cannot cause infection. During the period when the plant is receiving the greatest quantity of gelling saliva it is never infected. Much of this saliva is deposited in the mesophyll, whereas for infection the stylets must penetrate the phloem (Storey 1938). But I have evidence that 5 min. punctures often reach the phloem, and that saliva is deposited there. If then the gelling saliva is the vehicle of virus inoculation, the manner in which it so functions is still obscure.

In the experimental work here recorded I have received much assistance from Mr R. F. W. Nichols, which I gratefully acknowledge.

#### SUMMARY

When puncturing through a membrane into a fluid, *Cicadulina mbila* ejects saliva only when its stylets are in motion, and not when they are at rest and fluid is being drawn into them. This saliva sets to a gel and is moulded internally by the stylets to form a sheath. No other material of insect origin has been observed to flow from the stylets.

The salivary glands of infective insects, when inoculated into the abdomens of non-infective ones, caused a few of these to become infective. Comparative experiments with other organs from the insect support the interpretation that the salivary glands may contain virus, either in small quantities or occasionally.

Attempts to demonstrate virus in a fluid upon which many infective insects had fed were almost always negative. Only when infective and non-infective insects fed simultaneously on a film of fluid held between two membranes did a few of the non-infective insects become infective, and these never caused infection more than once in a series of tests.

Results were similar with a maize leaf on which infective insects had fed. Simultaneous feeding on a small area of leaf alone caused a few non-

infective insects to become infective, and these again were of low infective ability.

I suggest that an infective *Cicadulina mbila* ejects virus in very small quantities, so that only rarely can another individual take up enough ejected virus to make it infective and then only weakly so. The manner in which the virus is carried into the plant is still obscure; it is difficult to reconcile the view that the gelling saliva is the vehicle with the evidence of an earlier paper.

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#### DESCRIPTION OF PLATE 7

Photographs of the stylets of living *Cicadulina mbila*, puncturing through a paraffin membrane into a 10% sucrose solution, and of the salivary sheath formed during this process.  $\times 235$ .

FIG. 1. Maxillary stylets lying free in the fluid. By dark-field illumination.

FIG. 2. Early sheath formation. Stylets withdrawing leaving a thin sheath beyond their tip.

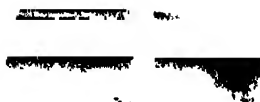
FIG. 3. Later sheath formation. Stout sheath formed with several droplets of saliva retaining their spherical form. One droplet of saliva just ejected at tip. The stylets were withdrawing during the exposure of the photograph.

FIG. 4. Stylets with large aggregation of Indian ink particles drawn to their tip. Two particles on the left were moving in during the exposure of the photograph and appear elongated in consequence.

FIG. 5. Stylets in resting position, partly withdrawn down the sheath. The end of the sheath is closed by a plug of saliva ejected at the moment of withdrawal.



1



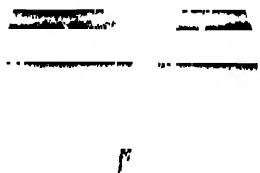
2



3



4



5



6



7



8



9



FIG. 6. A puncture close to the bottom of the observation chamber. Before the puncture was made the bottom bore a uniform layer of the heavier Indian ink particles that had settled naturally. Upon the first ejection of saliva, the particles were swept back well beyond the limits of the salivary mass, which lay in the middle of a clear area. Before the photograph was taken the insect had sucked twice, drawing in the two masses of particles that are visible near the end of the sheath. The stylets lie partly withdrawn in the sheath.

FIG. 7. An unbranched sheath in which the stylets had been resting with the distal end of the sheath plugged. On a sudden withdrawal of the stylets, air entered the sheath through the hole of puncture. The air bubble is visible at the base of the sheath.

FIG. 8. Abnormal formation of solid "strings" of saliva, due to the original salivary mass becoming detached from the membrane and so remaining unpenetrated by the advancing stylets. A normal tubular sheath can be seen on the left-hand side.

FIG. 9. Sheath with many terminal branches formed in fluid at pH 10.3.

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576.858.8:595.762

A comparative study of the transmission of *Hyoscyamus* virus 3, potato virus Y and cucumber virus 1 by the vectors *Myzus persicae* (Sulz), *M. circumflexus* (Buckton), and *Macrosiphum gei* (Koch)

By M. A. WATSON AND F. M. ROBERTS

*Rothamsted Experimental Station, Harpenden, Herts*

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[Plate 8]

1. INTRODUCTION

Insect transmitted plant viruses may be divided into two groups according to their relationships with their insect vectors. The first, which may be called the "Persistent viruses", survive in their vectors for long periods, sometimes for weeks or months; the second, or "Non-persistent viruses", survive in their vectors for only a short period, always less than that during which they remain active in untreated infective plant sap. There are other differences between the two types of viruses besides those of persistence in the vectors, and an attempt has been made in Table I to indicate the pro-

TABLE I. INSECT-TRANSMITTED VIRUSES GROUPED ACCORDING TO THEIR RELATIONSHIPS WITH THEIR INSECT VECTORS

Host	Virus	Class of vector	Sap transmission	Thermal inactivation		Survival in vectors	"Latent" period
				<i>in vitro</i> °C	<i>in vivo</i> sap		
Persistent viruses							
Aster	Yellows	Jassid	No	—	Indefinite	10 days	Kunkel 1926
Cotton	Leaf curl	White fly	No	—	7 days at least	6 hr.	Kirkpatrick 1931
Hemp	Bunchy-top	Aphis	No	—	—	2 days	Ofernia 1930
Maize	Streak and mottle	Jassid	No	—	3 weeks	1-2 days	Storey 1938
Pine-apple	Yellow-spot	Thrips	No	—	—	10 days	Linford 1932
Potato	Leaf roll	Aphis	No	—	10 days at least	55 hr.	Smith 1931
Potato	Yellow dwarf	Jassid	Yes	50	12 hr.	6 months	Black 1938
Pea	Pisum virus 1	Aphis	Yes	64	4 days	21 days	Osborn 1938
Peach	Yellows	Jassid	No	—	Several weeks	10 days	Hartzell 1936
Rice	Stunt	Jassid	No	—	"	3 days	Fukushu 1935
Sugar beet	Curly top	Jassid	Yes	76	7 days	"	(Bennett and Wallace 1938)
Sugar beet	Virus 3	Tingid	No	—	Several months	Not known	(Bennett 1935)
Tomato	Spotted wilt	Thrips	Yes	42	5 hr.	Indefinite	Kaufmann 1936
Raspberry	Yellow mosaic	Aphis	No	—	48 hr.	Probably none	Bald and Samuel 1931
Raspberry	Red mosaic	Aphis	No	—	12 hr.	Possibly 6 hr.	Bennett 1932
Non-persistent viruses							
Pea	Enation Mosaic	Aphis	Yes	62	4 days	12 hr.	Osborn 1937
Lettuce	Mosaic	Aphis	Yes	54	2 days	Short	* Ainsworth and Ogilvie 1939
Onion	Yellow dwarf	Aphis	Yes	Not known	Short	None	Drake, Tate and Harris 1932
Tobacco	Severe etch	Aphis	Yes	58	9 days	Short	Kassanis 1939
Cucumber	Virus 1	Aphis	Yes	60	2 days	8 hr.	Ainsworth 1935
Potato	Virus Y	Aphis	Yes	60	2 days	12 hr.	Smith 1931
Potato	Virus A	Aphis	Yes	50	24 hr.	Short	* Loughane 1933
<i>Hyocymamus</i>	Virus 3	Aphis	Yes	58	4 days	12 hr.	Hamilton 1932
Celery	Celery western mosaic	Aphis	Yes	56	6 days	10 hr.	Severin and Freitag 1938

\* Also private communication from the authors.



perties and range of vectors of the viruses in each group. The table is necessarily incomplete, for the properties of many viruses which are known to be insect transmitted, particularly those of the non-persistent type, have not yet been fully investigated.

The persistent viruses are rather variable in their general properties. Some can be mechanically transmitted, though usually with difficulty, while others can be transmitted only by grafting or by their insect vectors. The properties *in vitro* of the persistent viruses also vary. Curly top of sugar beet has been shown by Bennett (1935) to survive for several days in expressed sap, and to be resistant to chemical treatment, while spotted wilt virus of tomato (Bald and Samuel 1931, 1934) is very unstable, and survives in untreated sap for only a few hours. Within the persistent group there is also considerable variety of vectors, belonging to many families of the Hemiptera. These insects usually have a non-infective period after feeding on the infected plants which is known as the "latent" or "incubation" period. To account for the very high level of efficiency exhibited by these vectors some workers postulate that the viruses multiply in the bodies of the insects, thus maintaining the high virus content assumed necessary for the infection of successive healthy plants. The "latent" period is explained as being the time taken for the virus imbibed by the insects to reach an "infective" concentration. As, however, there is no information as to the size of an infective dose of virus, as delivered by an insect, or the number of infective doses which it could contain, this assumption is by no means essential. It is rendered less probable by the fact that the "latent" period is not decreased by increasing the quantity of virus available to the insects; for instance by prolonging the period of feeding on the infected plants (Freitag 1936). Also the facts that the degree of vector efficiency, and the length of time for which the vectors remain infective, are increased by increasing the feeding time on the infected plants, in those insects for which this treatment has been tested. Their efficiency therefore seems to depend rather on the quantity of virus which they acquire on feeding than on any subsequent process.

Although in Table I the non-persistent viruses are fewer in number than the persistent it is highly probable that there are, in fact, more of the non-persistent than of the persistent type. There are many insect-transmitted viruses whose physical properties agree with those of the non-persistent type, but whose insect-virus relationships have not yet been determined. The number of non-sap-transmissible viruses, or those which are difficult to transmit mechanically, whose vector relationships are unknown is much smaller. This comparative neglect of the non-persistent viruses is easily

accounted for. When viruses are difficult to transmit mechanically, understanding of the insect-virus relationships is essential to easy propagation of the viruses, and no progress can be made without it. When the viruses are readily sap-transmissible their properties can more easily be determined without the use of their vectors. Also the specific and obligate nature of the insect transmission observed for the persistent viruses has led workers to consider it as of particular complexity and interest whereas the superficial nature of the studies which have so far been made on the non-persistent type has led some workers to the conclusion that they are of minor interest. Other reasons for the lack of information on the insect-virus relationships of the non-persistent viruses are that the vectors lose their infectivity rapidly, which makes complex experimentation difficult and tedious, and also lack of knowledge of the conditions necessary for optimum infectivity of the insects has led either to the use of large, unwieldy numbers of insects, or else to small and inadequate data.

On the whole the non-persistent viruses, so far as their properties have been determined, form a more homogeneous group than the persistent types. Their thermal death points and times of survival in expressed sap lie within a comparatively small range, and all are transmitted by a single family of the Hemiptera, namely the Aphididae. The vectors become capable of transmitting the viruses almost immediately after contact with the source of infection, and there is no indication of a "latent" period between the infection feeding and effective transmission. This property, combined with the fact that the virus survives for only a short time in the fasting aphides, and an even shorter time if they are fed on a succession of healthy plants (Watson 1936; Osborn 1937) suggested to many workers that the transmission of the non-persistent viruses was effected mechanically, merely by contamination of the outside of the stylets. Recent work, however (Watson 1936, 1938a), has shown that the relationship of at least one non-persistent virus, *Hyoscyamus* virus 3, with its vector *Myzus persicae*, cannot be explained in this simple way. Hy 3 virus was found to possess two remarkable properties. The first is that the efficiency of the vector was greatly increased by even short periods of fasting before feeding on the infected plants, and the second is that the vector efficiency of the aphides declines with increasing feeding time on the source of infection. In both these properties Hy 3 virus differs from the persistent type of virus. It has already been noted that the efficiency and duration of infectivity in the vectors of the persistent viruses is increased by increasing the infection feeding time. The optimum efficiency of these vectors therefore follows a period of feeding and not of fasting.

Potato virus Y, and cucumber virus 1 behave in relation to their insect vectors in the same way as Hy 3 virus (Watson 1938*b*). Mr B. Kassanis, working in this department, has found that severe etch virus of tobacco is transmitted by aphides, and also responds to preliminary fasting of the vectors, and to variation in the infection feeding time in a similar manner to Hy 3. Thus four out of the eight non-persistent viruses behave similarly in relation to their vectors, and it is not unreasonable to suppose that many others do so.

As yet only aphid transmitted viruses have proved non-persistent, and none of the non-persistent viruses studied differ greatly from each other. There may of course be viruses which will not fit into the two groups defined in this paper. The red and yellow mosaics of raspberry (Bennett 1932) apparently possess some properties pertaining to both groups. Also there may be non-persistent viruses which do not show the preliminary fasting and other effects which distinguish the viruses already studied, and such viruses might prove to be transmitted by purely mechanical means. But the fact that the non-persistent viruses so far studied are similar in their properties, and that the vectors are all Aphididae suggests that their transmission is not simply a fortuitous contamination of the insect's stylets. Most of the evidence indicates that transmission of the non-persistent viruses is, on the other hand, a special attribute of a particular type of virus and vector and is probably a complex and specialized process. This hypothesis accords with several other facts not explained by the hypothesis that the non-persistent viruses are transmitted by simple contamination of the stylets. The highly infectious viruses such as tobacco mosaic, cannot be transmitted by insects which readily transmit much less infectious and less stable viruses. This is understandable if the non-persistent viruses possess special properties which are independent of their transmissibility by mechanical inoculation.

## 2. METHODS

The methods used in these experiments were similar to those previously described (Watson 1936, 1938*a*), but the necessity for repeating each operation with several species of aphides or strains of viruses, some of which required several aphides per plant to give adequate numbers of infections, made it impossible to use very accurate methods for controlling the times of feeding. Aphides fed in an atmosphere of high humidity nearly all penetrate the leaves within 2 or 3 min. of being placed on them, and the difference between the effects of 2 and 4 min. feeding was small compared

with that between 2 and 15 min., or longer periods. In the so-called "2 min." feeding groups of aphides were placed on the leaves in optimum feeding conditions, and removed after 5 min. Aphides not feeding in this time were not used. The 1 and 5 hr. feedings were also approximate though the 1 hr. feeding and fasting periods were never less than 1 hr. The loss of accuracy due to such variations was probably very small. Tobacco, being a common host of all the viruses was used in comparative experiments.

### 3. VIRUSES

The viruses used were (a) *Hyoscyamus* virus 3, in three strains isolated by single aphides from the parent strain (b) potato virus Y, of one strain only, which was obtained from commercially grown *Hyoscyamus*, and Hamilton described under the name of *Hyoscyamus* virus 2 (1932), and (c) cucumber virus 1 in two strains, originally isolated by Price (1934) and kindly supplied to the author by Dr Kenneth Smith.

Although these viruses are similar in many of their properties they appear to be unrelated. Inoculation of tobacco plants with potato virus Y, or cucumber virus 1 does not protect them against reinoculations with Hy 3 virus, nor did previous infection with potato virus Y render them immune to reinoculation with cucumber virus 1. Neither potato virus Y nor cucumber virus 1 showed any precipitate when mixed with anti-serum prepared against Hy 3 virus, and potato virus Y anti-serum did not react with solutions of Hy 3 virus or cucumber virus 1.

Two strains of Hy 3 virus used in addition to the parent strain have been called "weak" Hy 3, or Hy 3W, and yellow-spot virus, or Hy 3S. The parent strain is distinguished as "virulent" Hy 3, or Hy 3V (fig. 1, Plate 8).

Hy 3W is avirulent towards tobacco, causing only a faint green mottle of the "vein-band" type (fig. 2, Plate 8). No symptoms are produced until the plants have been infected for at least 10 days, and in the very mild forms isolated by single aphides the symptoms may appear only after 3 or 4 weeks. Freshly extracted sap from such plants does not precipitate with Hy 3 anti-serum, which readily causes precipitation of freshly extracted sap from Hy 3V plants. When the sap from the Hy 3W plants is concentrated by repeated precipitations with ammonium sulphate and re-solution in a considerably smaller volume of water, it gives a precipitate corresponding to that obtained with diluted sap from Hy 3V plants.

Plants could be protected from infection with Hy 3V virus by previous inoculation with Hy 3W if the symptoms of the latter were well established, but this protection was incomplete, for local symptoms of the virulent strain could be obtained in any part of the plant not actually showing symptoms of Hy 3W. Hy 3V always eventually became systemic, though this sometimes took 6 or 8 weeks, and the resulting infection was less severe than when the healthy plants were inoculated with Hy 3V only. Even Hy 3W infected plants which were not infected with Hy 3V showed isolated yellow patches of the virulent strain after many weeks of apparently unmixed infection, and later the symptoms of Hy 3V became systemic, giving an appearance similar to that of mixed inoculation (fig. 3, Plate 8). In the early stages Hy 3W could be recovered from such plants by careful isolation of the green areas, or by the use of single aphides, which on the few occasions when they did transmit the virus appeared to carry only one strain at a time. Plants infected by aphides rarely showed intermediate symptoms, such as were produced either by reinoculation with the virulent strain or by spontaneous appearance of Hy 3V in the Hy 3W plants, or by inoculation with a mixture of infected saps consisting of equal quantities of untreated Hy 3W sap and Hy 3V sap diluted to 1/10,000.

Hy 3S was called the "yellow-spot" strain because of its very distinctive symptoms (fig. 4, Plate 8). These took the form of isolated bright yellow spots on the inoculated leaves and bright yellow flecks on most of the other leaves. The older leaves generally became completely chlorotic and were difficult to distinguish from similar leaves of Hy 3V infected plants. The youngest leaves showed no, or few, yellow flecks and contained very little virus. Sap taken from the chlorotic leaves, or from those with a large number of yellow flecks precipitated readily with Hy 3V anti-serum, but the plants as a whole contain less virus (see Table XVII), than Hy 3V infected plants. Aphides that had fed on the yellow patches were very infective, but in practice several aphides were used for each healthy plant so as to be reasonably certain that one of the group would have fed on an infective area. With Hy 3W, however, single aphides were more satisfactory in comparative experiments, because of the danger of the weak strain being masked by infection from aphides which had fed on parts of the leaf developing local symptoms of Hy 3V. This necessitated the use of so many healthy plants as to preclude the inclusion of Hy 3W in the large comparative experiments.

The two strains of cucumber virus used have been described by Price (1934). The green strain, cucumber virus 1G appears to be avirulent towards tobacco the symptoms being inconspicuous, especially in older

plants which have rather narrow deep green leaves with a rough leathery texture. The yellow strain of cucumber virus 1, strain 1 Y, gave much more conspicuous symptoms: large, pale, or yellowish areas on the leaves, the green areas being sometimes deformed. Actually there was less virus per plant in the cucumber 1 Y infected plants than in those infected with cucumber virus 1 G (see Table XVII) and the yellow strain was much the most difficult to transmit by means of aphides. The two strains appeared to be quite stable and independent. The green areas on chlorotic leaves from Y infected plants did not contain strain G, but strain Y could sometimes be isolated from small yellow patches which occasionally appeared on G infected plants.

#### 4. DESIGN OF EXPERIMENTS

As in previous experiments (Watson 1936, 1938*a*) factorial arrangements (Fisher 1934) were used, in which all combinations of a number of groups of treatments were compared. These groups of treatments consisted of

- (1) Viruses,
- (2) Vectors,
- (3) Preliminary fasting times,
- (4) Feeding times on the infected plants.

These groups could not all be included in every experiment and for those which contained several species of aphides and several viruses, only one variable treatment such as preliminary fasting or infection feeding time was given. The times of preliminary fasting and infection feeding used were determined from the results of previous experiments, in which a wider range of times had been tested. The times used were: preliminary fasting—none, 1 hr., and 4 or 5 hr.; infection feeding times—2 min., 15 min., and 1 or more hours. The number of hours used for the longest infection feeding times was a matter of convenience, for although the relation between infection feeding time and percentage infection was of the same type in all experiments, the position of the point of minimum infectivity varied. All experiments were repeated from seven to ten times at weekly intervals. Although the level of infectivity varied between occasions the differences between treatments were usually similar on all occasions. The single outstanding exception is noted later.

Two types of experiment were used. (1) Comparisons were made between viruses and between vectors, and only one type of feeding treatment was varied (experiments I, II, Va and VI). (2) The effects of both preliminary fasting and infection feeding time were determined for a single virus and

a single vector (experiments III, IV and Vb). In experiments of the second type, a control consisting of Hy 3V virus transmitted by *Myzus persicae* and receiving only one treatment from the main experiment, was also carried out, so as to form an independent check on the conditions affecting the infectivity of the vectors.

In the first type of experiment particularly, the number of aphides used per plant for the less virulent strains and less efficient vectors were arranged so as to give numbers of infections comparable with those obtained for Hy 3V virus and *M. persicae*, so as to ensure that all treatments should have comparable standard errors. The number of aphides used per plant was, however, limited by the speed with which they could be manipulated, for with increasing numbers it became more difficult to time the treatments accurately. With very poor vectors, therefore, or viruses transmitted with difficulty, the numbers of infections obtained were often very small, although the number of aphides used per plant was increased to the practicable limit.

The standard errors obtained from statistical analysis of the results are not given in the tables because the analyses were not done on the actual results but on the angular transformations of the figures which give a better estimate of the errors involved (Fisher and Yates 1938).

The experiments are not given in chronological order but in the order most convenient for discussion. Some of the arrangements used in those described first are based on information contained in experiments given later in the paper. The time of year at which they were made is given in the descriptions of the tables. In the descriptions of individual experiments only a very brief account of the significant results is given. The main effects and interactions are discussed collectively under separate headings.

## 5. EXPERIMENTS WITH *MYZUS PERSICAE* AND *MYZUS CIRCUMFLEXUS*

### *Experiment I.*

#### Variable factors:

- (1) Aphides: *M. persicae*, *M. circumflexus*.
- (2) Viruses: Hy 3V and Hy 3S, potato virus Y, cucumber virus 1G.
- (3) Preliminary fasting times: none, 1 hr., 5 hr.

#### Constant factors:

- (1) Feeding time on infected plants: 2 min.
- (2) Feeding time on healthy plants: 20 hr.

In experiments II and VI the same strains of viruses were used in testing the different aphides. In experiment I two different strains of Hy 3, namely, Hy 3V and Hy 3S, were used in testing respectively *M. persicae* and *M. circumflexus*. Hy 3S was used in the *circumflexus* section of this experiment because the information was needed for this particular strain, and had already been obtained for Hy 3V. The results of experiment I are given in Table II.

TABLE II. EXPERIMENT I. EFFECT OF PRELIMINARY FASTING ON THE TRANSMISSION OF HY 3 VIRUS, POTATO VIRUS Y AND CUCUMBER VIRUS 1G, BY *M. PERSICAE* AND *M. CIRCUMFLEXUS*

Five plants per treatment; repeated on ten occasions; September to December. Total, 50 plants per treatment.

Vectors	Viruses	No. of aphides per plant	Preliminary fasting times			Total
			None	1 hr.	5 hr.	
<i>M. persicae</i>	Hy 3V	1	7	26	38	61
	Potato Y	1	2	17	31	50
	Cucumber 1G	3	5	10	15	30
	Total		14	53	84	141
<i>M. circumflexus</i>	Hy 3S	3	4	11	20	35
	Potato Y	1	2	3	11	16
	Cucumber 1G	3	4	8	13	25
	Total		10	22	44	76
	Total		24	75	128	217

For all aphides and viruses the infectivity increased considerably with increasing time of preliminary fasting. The two aphides differed in vector efficiency, and this varied with the different viruses and on different occasions.

### Experiment II.

#### Variable factors:

- (1) Aphides: *M. persicae*, *M. circumflexus*.
- (2) Viruses: Hy 3V, potato virus Y, cucumber virus 1G.
- (3) Infection feeding times: 2 min., 15 min., 4 hr.

#### Constant factors:

- (1) Preliminary fasting time: 1-2 hr.
- (2) Feeding time on healthy plants: 20 hr.

The results are given in Table III.



TABLE III. EXPERIMENT II. EFFECT OF VARYING INFECTION FEEDING TIMES ON TRANSMISSION OF HY 3V VIRUS, POTATO VIRUS Y AND CUCUMBER VIRUS 1G, BY *M. persicae* AND *M. circumflexus*

Five plants per treatment; repeated on nine occasions; March to May. Total, 45 plants per treatment.

Vectors	Viruses	No. of aphides per plant	Infection feeding times			Total
			2 min.	15 min.	4 hr.	
<i>M. persicae</i>	Hy 3V	1	34	18	5	57
	Potato Y	1	25	15	5	45
	Cucumber 1G	3	19	19	1	39
	Total		78	52	11	141
<i>M. circumflexus</i>	Hy 3V	1	21	10	2	33
	Potato Y	1	9	5	0	14
	Cucumber 1G	3	39	25	3	67
	Total		69	40	5	114
	Total		147	92	16	255

Increase in the times of feeding on the infected plant decreased the vector efficiency of both aphides with all the viruses. As in Exp. I the vector efficiency of the different aphides differed as did the infectivity of the viruses. The difference in vector efficiency between aphides was least for cucumber virus 1G.

Before most of the experiments with vectors and viruses whose properties were unknown, tests were carried out to determine the numbers of aphides per plant needed to give adequate numbers of infections. With the other viruses these tests were comparatively short and simple as the viruses were readily transmissible by small numbers of aphides. The large numbers needed to transmit cucumber virus 1Y, however, made the preliminary tests with this virus rather more elaborate, and it is interesting to compare the results with those of previous tests made in a similar way with Hy 3V virus and *M. persicae* (Watson 1936). In the previous experiments aphides fed on virus infected plants were placed on the healthy plants in groups of 1, 5, 10 and 20 aphides. From the results a maximum likelihood value was obtained for the probability of infection by a single aphid, on the assumption that the infections were local and independent. Values thus calculated for the other numbers of aphides used agreed closely with the actual results of the experiment. The results of the experiment therefore agreed with the hypothesis that the infections were local and independent, and not that they were the result of accumulation in the healthy plants of small, subinfective doses of viruses, injected by insects which individually would not have caused infection.

TABLE IV. DISTRIBUTION OF INFECTION FOR APHID GROUPS OF VARYING NUMBER. CUCUMBER VIRUS 1Y TRANSMITTED BY *M. PERSICAE*

No. of aphides per plant ...	1	5	10	20
No. of plants infected out of 90	1	6	15	26
Expected number if $1 - Q = 0.017$	2	7	14	26

$1 - Q$  = probability of infection by a single aphid.

The results of a similar experiment with cucumber virus 1Y are given in Table IV. They differ from the Hy 3V results previously obtained in that only one insect out of 90, in the single aphid group, succeeded in transmitting the virus. It might be supposed that this was merely an "accidental" infection, and that one aphid being unable to take up enough virus to cause infection, several infective aphides together were necessary, i.e. that the infections were cumulative, and not independent of each other. However, if the figures be examined by the method used for the 1936 experiment, no real difference is found between the two sets of data. The estimated probability of infection by cucumber virus 1Y for a single aphid is 0.017, that is, 17 aphides in every thousand carry an infective dose. The numbers of infections obtained by larger numbers of aphides on smaller numbers of plants can be calculated from this figure, assuming that the infections are local and independent. The numbers expected for the numbers of aphides and plants used in the experiment are given in the second line of Table IV, and do not differ significantly from the figures actually obtained; there is no reason to doubt the truth of the hypothesis of local and independent infection. In addition to this, as in the 1936 experiments, a further test can be applied. If the aphides were capable of causing infection by the introduction into the plants of a number of subinfective doses of virus, then as the aphid number increased smaller and smaller quantities of virus would be utilized in causing infection. Aphides which contained insufficient virus to infect a plant in a group of five, might do so in a group of ten, and so the figures actually obtained with the higher numbers of aphides would be considerably above those estimated on the assumption that the infections are local and independent. In actual fact the highest figures obtained were a fraction smaller than the estimated figures, though not significantly different. The possibility of infection being caused by accumulation of subinfective doses is therefore remote. Storey (1938) has found a similar result for experiments on the transmission of streak disease of maize by *Cicadulina mbila*.

As a preliminary to determining the infectivity of cucumber virus 1Y with different vectors and in varying conditions the results of this experi-

ment were not very promising, for it was carried out in conditions of preliminary fasting and infection feeding, which were optimal for *Myzus persicae*—so far the most efficient of the three vectors. It was obvious that at least 100 aphides would be needed on each occasion, for each treatment, to ensure infection in optimum conditions even with *M. persicae*, and this number was impracticable for use in more complex experiments. Some attempts were made to include cucumber virus 1Y in the comparative experiments with smaller aphid numbers but the number of infections obtained was almost negligible. The only adequate results with this virus were obtained in experiment III, which had the following treatments:

Variable factors:

- (1) Preliminary fasting times: none, 1 hr., 4 hr.
- (2) Infection feeding times: 2 min., 15 min., 4 hr.

Constant factors:

- (1) Vector: *M. persicae*.
- (2) Virus: Cucumber virus 1Y.
- (3) Feeding time on healthy plants: 20 hr.

The 100 aphides used for each treatment were distributed over ten plants per treatment in groups of ten. The results are given in Table V.

TABLE V. EXPERIMENT III. EFFECT OF PRELIMINARY FASTING, AND INFECTION FEEDING TIMES, ON THE TRANSMISSION OF CUCUMBER VIRUS 1Y, BY *M. PERSICAE*

Ten aphides per plant; ten plants per treatment repeated on six occasions; January and February. Total, 60 plants per treatment.

Preliminary fasting times	Infection feeding times			Total
	2 min.	15 min.	4 hr.	
None	2	1	0	3
1 hr.	2	5	0	7
4 hr.	3	7	1	11
Total	7	13	1	21

Allowing for the difference in transmissibility of the virus, and the consequent increase in the experimental error, the results of experiment III agreed with those obtained for cucumber virus 1G in experiments I and II. The vector efficiency of the aphides increased with increasing preliminary fasting times, and decreased with increasing infection feeding times. As with cucumber virus 1G the relative rate of decrease for infection feeding times differed from that of the other viruses. These differences will be discussed later.

*Experiment IV.*

## Variable factors:

- (1) Preliminary fasting: none, 1 hr., 4 hr.
- (2) Infection feeding times: 2 min., 15 min., 4 hr.

## Constant factors:

- (1) Vector: *M. persicae*.
- (2) Virus: Hy 3S (yellow-spot strain).
- (3) Feeding time on healthy plants: 20 hr.

This experiment was designed to show the effect of the standard preliminary fasting and infection feeding treatments on the virus when transmitted by *M. persicae*. The relationship of Hy 3S to the other viruses and vectors were partly determined by including it in experiment I. The results of experiment III are given in Table VI.

TABLE VI. EXPERIMENT IV. EFFECT OF PRELIMINARY FASTING AND INFECTION FEEDING TIMES ON TRANSMISSION OF HY 3S VIRUS, BY *M. PERSICAE*

Five aphides per plant; five plants per treatment, repeated on ten occasions; June–September. Total, 50 plants per treatment.

Preliminary fasting times	Infection feeding times			Total
	2 min.	15 min.	4 hr.	
None	5	8	9	22
1 hr.	28	15	7	50
4 hr.	31	24	13	68
Total	64	47	29	140

As five aphides were used per plant large numbers of infections were obtained for the optimum treatments. The effects of varying preliminary fasting and infection feeding times were very similar to those obtained with Hy 3V virus when transmitted by *M. persicae*.

## 6. EXPERIMENTS WITH *MYZUS PERSICAE* AND *MACROSIPHUM GEI*

The original purpose of the experiments with *Macrosiphum gei* was to compare its efficiency in transmitting the different viruses with that of other vectors using all the viruses with variations in both preliminary fasting times and infection feeding times. But *M. gei* seemed to reproduce more slowly than the other aphides, and so many were required for each experiment that the stock cultures became reduced and first one and then another section had to be discontinued. The results from this incomplete series of comparisons are presented in two parts, experiments V and VI.

Experiment V contains results from those sections for which variations in both preliminary fasting and infection feeding were given. Experiment VI contains the sections in which only infection feeding was varied, and the preliminary fasting time was constant. Experiment VI also includes results for the corresponding preliminary fasting times from experiment V. The data from each experiment are further subdivided so that for wider ranges of treatment the tables include only strictly comparable results, but where some treatments have been repeated more frequently than others, all the data are included. Thus experiment VA contains the data for Hy 3 virus and potato virus Y compared as to the effects of preliminary fasting and infection feeding on only four occasions. Experiment VB gives the totals for seven repetitions of the same treatments for Hy 3 virus only.

The treatments used in Exp. V are as follows:

Table VII. EXPERIMENT VA. EFFECT OF PRELIMINARY FASTING AND INFECTION FEEDING TIMES ON THE TRANSMISSION OF HY 3V VIRUS AND POTATO VIRUS Y BY *MACROSIPHUM GEI*

Three aphides per plant, five plants per treatment repeated on four occasions; May-June. Total, 20 plants per treatment.

Preliminary fasting times		Infection feeding times			Total
		2 min.	15 min.	5 hr.	
Hy 3V virus	None	1	0	0	1
	1 hr.	3	1	1	5
	5 hr.	4	1	2	7
	Total	8	2	3	13
Potato virus Y	None	2	3	1	6
	1 hr.	8	3	0	11
	5 hr.	7	1	1	9
	Total	17	7	2	26

Table VIII. EXPERIMENT VB. EFFECT OF PRELIMINARY FASTING AND INFECTION FEEDING TIMES ON THE TRANSMISSION OF HY 3V VIRUS BY *MACROSIPHUM GEI*

Three aphides per plant; five plants per treatment, repeated on seven occasions including four from Table VII. May-July. Total, 35 plants per treatment.

Preliminary fasting times	Infection feeding times			Total
	2 min.	15 min.	4 hr.	
None	1	0	0	1
1 hr.	7	1	1	9
3 hr.	7	2	2	11
Total	15	3	3	21

## Variable factors:

- (1) Viruses: Hy 3V, potato virus Y.
- (2) Preliminary fasting times: none, 1 hr., 4 hr.
- (3) Infection feeding times: 2 min., 15 min., 3 hr.

## Constant factors:

- (1) Vector: *Macrosiphum gei*.
- (2) Feeding time on healthy plants: 20 hr.

The results are given in Tables VII and VIII.

*M. gei* showed an increase in vector efficiency with increasing preliminary fasting time up to 1 hr. and a rapid decrease with increasing infection feeding times from 2 to 15 min. In these relative rates of increase and decrease of vector efficiency *M. gei* seems to differ slightly from the *Myzus* species.

*Exp. VI.*

## Variable factors:

- (1) Aphides: *Macrosiphum gei*, *Myzus persicae*.
- (2) Viruses: Hy 3V, potato virus Y, cucumber virus 1 G.
- (3) Infection feeding times: 2 min., 15 min., 5 hr.

## Constant factors:

- (1) Preliminary fasting: 1 hr.
- (2) Feeding time on healthy plants: 20 hr.

The results of this experiment are given in Tables IX, X and XI.

TABLE IX. EXPERIMENT VIA. EFFECT OF VARYING INFECTION FEEDING TIMES ON THE TRANSMISSION OF HY 3V VIRUS, POTATO VIRUS Y AND CUCUMBER VIRUS 1G, BY *MYZUS PERSICAE* AND *MACROSIPHUM GEI*

Five plants per treatment; seven occasions. May-July. Total, 35 plants per treatment.

Vectors	Viruses	No. of aphides per plant	Infection feeding times			Total
			2 min.	15 min.	5 hr.	
<i>Myzus persicae</i>	Hy 3V	1	21	11	3	35
	Potato Y	1	16	7	2	25
	Cucumber 1G	3	11	8	1	20
	Total		49	26	6	80
<i>Macrosiphum gei</i>	Hy 3V	3	5	1	1	7
	Potato Y	3	7	3	2	12
	Cucumber 1G	3	2	0	0	2
	Total		14	4	3	21
Total			63	30	9	101

TABLE X. EXPERIMENT VIb. TRANSMISSION OF HY 3V VIRUS AND POTATO VIRUS Y. TREATMENTS AS IN TABLE IX, AND DATA FROM TABLE IX INCLUDED

Nine repetitions; May–July. Total, 45 plants per treatment.

Vectors	Viruses	No. of aphides per plant	Infection feeding times			Total
			2 min.	15 min.	5 hr.	
<i>Myzus persicae</i>	Hy 3V	1	26	15	3	44
	Potato Y	1	18	10	2	30
	Total		44	25	5	74
<i>Macrosiphum gei</i>	Hy 3V	3	7	1	1	9
	Potato Y	3	12	4	2	18
	Total		19	5	3	27
	Total		64	30	8	102

TABLE XI. EXPERIMENT VIc. TRANSMISSION OF HY 3V VIRUS BY *MACROSIPHUM GEI*. INCLUDING DATA FROM TABLE IX. OTHER TREATMENTS AS IN TABLE IX

Twelve repetitions; May–August. Total, 60 plants per treatment.

Aphides	No. of aphides per plant	Infection feeding times			Total
		2 min.	15 min.	5 hr.	
<i>Myzus persicae</i>	1	39	21	5	65
<i>Macrosiphum gei</i>	3	11	2	2	15
Total		50	23	7	80

The vector efficiency of *Macrosiphum gei* was always much lower than that of the *Myzus persicae* controls, and, as with *M. persicae*, transmission of cucumber virus 1G was very much less efficient than that of Hy 3V. Between Hy 3V and potato virus Y, however, the positions were different, for potato virus Y, which is less readily transmitted by *M. persicae* and *M. circumflexus* than is Hy 3V virus, seemed to give more infections than Hy 3V when transmitted by *Macrosiphum gei*. The difference is not significant, but even if the number of infections obtained by *M. gei* with Hy 3V virus and potato virus Y were equal, this is still contrary to the findings for the other vectors.

## 7. EXPERIMENTS WITH HY 3W VIRUS

Reasons have already been given for the non-inclusion of Hy 3W in the complex experiments. The results given in Table XII are taken from a number of isolated experiments, in some of which shorter preliminary fasting, or longer infection feeding times were given in addition to those

included in the table, but which gave no infections. The indications are that the response of Hy 3W to these treatments is similar to that of Hy 3V and Hy 3S, but a very large experiment would have to be carried out to determine its magnitude accurately.

TABLE XII. APHID TRANSMISSION OF HY 3W VIRUS, WITH VARYING CONDITIONS AND APHID NUMBER

Virus	Aphid	No. of aphides	No. of plants used	Pre-liminary fasting	Infection feeding	No. of infections
Hy 3W,	<i>Myzus persicae</i>	2	30	3 hr.	2 min.	2
Hy 3V	"	1	30	3 hr.	2 min.	21
Mixed infection	"	1	30	3 hr.	2 min.	{ 13 Hy 3V, 1 Hy 3W
Hy 3W and Hy 3V }						
Hy 3W	<i>M. persicae</i>	3	15	1 hr.	2 min.	2
"	"	3	15	1 hr.	15 min.	1
"	"	3	15	1 hr.	3 hr.	0
Hy 3W	<i>M. circumflexus</i>	3	15	1 hr.	2 min.	0
"	"	3	15	1 hr.	15 min.	0
"	"	3	15	1 hr.	3 hr.	0
Hy 3W	<i>M. persicae</i>	1	45	3 hr.	2 min.	1
"	"	2	15	6 hr.	2 min.	1

The main interest of the results given in Table XII lies in comparing them with the figures for transmissibility of Hy 3V virus and with the infectivity of both strains as determined by local lesion counts (see § 6). No comprehensive studies have been made on the transmissibility of Hy 3W virus by *Myzus circumflexus* and *Macrosiphum gei* but both these vectors are very inefficient.

## 8. INDIVIDUAL TREATMENT EFFECTS

### (a) Preliminary fasting times

An increase in vector efficiency corresponding with increase in the times for which the aphides fasted before feeding on the infected plants was shown by all the aphides in their transmission of all the viruses. Table XIII gives the results for the different experiments with each virus and vector, and the means for all experiments where more than one was made on the same virus and vector. The results are expressed as percentages of plants infected, and where more than one aphid per plant was used the percentage infection by a single aphid per plant has been calculated, so that the comparisons could be made on a uniform basis. Some of the data for Hy 3V



TABLE XIII. EFFECT OF TIME OF INFECTION FEEDING COMPARED FOR ALL APHIDES AND VIRUSES. PERCENTAGE INFECTION BY ONE APHID PER PLANT

Where more than one aphid was used in the experiments, the percentage infectivity for one aphid per plant was calculated as  $100(1 - Q)$ , where  $Q^2$  is the observed probability of infection for  $X$  aphides per plant.

Viruses	Pre-liminary fasting	<i>Myzus persicae</i>					<i>Myzus circumflexus</i> (1939)	<i>Macrosiphum gei</i> (1939)
		Previous exps. (1938)		Present exps. (1939)			Exp. I	Exp. V
		III	IV	VIII	I	Mean		
Hy 3V	None	8	30	14	19	15	7	0.5
	1 hr.	42	53	52	53	50	19	4
	5 hr.	54	73	76	67	65	33	5
Hy 3S	None					Exp. IV	Exp. I	
	1 hr.					2	3	
	5 hr.					15	8	
Potato Y	None			Mitchell (1937)	Exp. I	Mean	Exp. I	Exp. V
	1 hr.			5	4	5	4	3
	5 hr.			22	34	27	6	9
Cucumber 1G	None			28	62	40	10	6
	1 hr.			Exp. VIII	Exp. I	Mean	Exp. I	
	5 hr.			2	3	3	3	
Cucumber 1Y	None			8	7	8	6	
	1 hr.			12	11	12	10	
	5 hr.					Exp. III		
Cucumber 1Y	None					0.2		
	1 hr.					0.8		
	5 hr.					1.2		

and potato virus Y belong to experiments the results of which have been published previously (Watson 1936, 1938a; Mitchell 1937), and one, experiment VIII, which has not been published in detail.

The results are taken from those sections of the experiments in which the optimum infection feeding times of 2 or 15 min. had been given.

Although in different experiments the percentage of infections for corresponding preliminary fasting time is variable, the relation between the percentage infections for different fasting time is fairly uniform for all viruses and for the vectors *Myzus persicae* and *M. circumflexus*. There is no recognized method which can be used for testing the uniformity of data

of this kind. Such statistical examination as has been attempted, e.g. by using a modification of the  $\chi^2$  test, have so far indicated that the data are, in fact, uniform, but no accurate estimate of the relations between the results for different preliminary fasting times have been made. The proportions suggested below are merely a convenient way of presenting the information obtained from a general survey of the results.

The increases from zero to 1 hr. fasting and from 1 to 5 hr. are related to each other in the approximate proportion 1 : 3 : 5. In some of the previous experiments a wider range of preliminary fasting times were used, and if these figures are included the proportion becomes:

Preliminary fasting times:	none	15 min.	1 hr.	4-6 hr.	12 hr.
Ratio of percentage infection:	1	2	3	5	5.5

The results for *Macrosiphum gei* when transmitting Hy 3V virus and potato virus Y, differ from those for *Myzus persicae* and *M. circumflexus* in that the relations between the results for the different preliminary fasting times are approximately in the proportion of 1 : 5 : 5 showing a more rapid increase from none to 1 hr. preliminary fasting, and none between 1 and 5 hr.

#### (b) Infection feeding time

The vector efficiency of all the aphides decreased with increasing feeding time on the infected plants if preliminary fasting had been given. The percentage infections for individual experiments, and the means for all experiments, are given in Table XIV. Like Table XIII, Table XIV contains data from some experiments published previously (1938a) and one set from an experiment carried out by Mitchell (1937). Where more than one aphid per plant was used the calculated probability of infection by one aphid per plant is given, as in Table XIII.

The difficulties of estimating the uniformity of the data and the relationship between the results for different infection feeding times are the same as for the preliminary fasting times, but as in the previous section there is one group of aphides and viruses for which the data appears to be uniform. The aphides are *M. circumflexus* and *M. persicae*, and the viruses Hy 3V and S and potato virus Y. For these vectors and viruses there seems to be a considerable loss of infectivity during the first 15 min. of feeding; between 15 min. and 1 hr. the decrease is less rapid. The closest approximation to the relation between the percentage infection for the three feeding times is 5 : 3 : 1. Previous experiments with Hy 3V and potato virus Y have shown that the percentage of infection does not decrease further if the feeding time is continued for longer periods than

1 hr. At this point, or sometimes a little later, the efficiency of the vectors seems to reach a permanent low level, or in some experiments (Watson 1936, 1938a) increased slightly when 6 or 12 hr. of infection feeding had been given. In these experiments the relation between the results for different infection feeding times was approximately as follows:

Infection feeding times: 2 min. 5 min. 15 min. 1 hr. 6 hr. 12 hr.  
Ratio of percentage infection: 5 4 3 1 1.5 2

TABLE XIV. EFFECT OF INFECTION FEEDING TIMES COMPARED FOR ALL APHIDES AND VIRUSES. PERCENTAGE INFECTIONS FOR ONE APHID PER PLANT, CALCULATED AS IN TABLE XIII

		<i>M. persicae</i>						<i>M. circum-</i>	<i>M. gei</i>			
		Previous expts.			Present expts.			<i>florus</i>	(1939)			
		(1938)			(1939)			(1939)				
Viruses	Infection feeding	(1936)	I	II	II	VI	Mean	Exp. II	Exp. V	Exp. VI	Mean	
Hy 3V	2 min.	59	72	65	65	75	66	47	6	7	7	
	15 min.	34	32	43	35	40	36	22	1	1	1	
	5 hr.	11	12	18	23	11	13	4	1	1	1	
							Exp. IV					
Hy 3S	2 min.						18					
	15 min.						12					
	5 hr.						4					
		(1939)										
		Mitchell										
		(1937)	Exp. II	Exp. VI	Mean	Exp. II	Exp. V	Exp. VI	Mean			
Potato Y	2 min.	33	55	40	38	20	10	14	12			
	15 min.	25	33	22	26	11	3	3	3			
	5 hr.	7	11	4	8	0	1	1	1			
		1939 expts.										
		II	VIa	VII	VIII	Mean						
Cucumber 1G	2 min.	17	12	12	8	12						
	15 min.	17	6	9	10	11						
	5 hr.	1	1	1	3	2						
							Exp. III					
Cucumber 1Y	2 min.						0.6					
	15 min.						1.2					
	5 hr.						0.1					

The outstanding exception among the viruses to this uniformity of response to varying infection feeding times was cucumber virus 1. When transmitted by *M. persicae* this virus sometimes failed to show any decrease in infectivity between 2 and 15 min. infection feeding. This effect can be seen for cucumber virus 1G in the results of experiment II, and in experiment VIII whose results are not given in detail. It appears in the analyses of variance as interaction between aphides and viruses, which is significant in experiment II. Even in this experiment, however, the results are not absolutely uniform, and on some occasions *M. persicae* showed with cucumber virus 1G a decrease in vector efficiency corresponding to that of *M. circumflexus*, which, throughout all the experiments, behaved with cucumber virus 1G, exactly as with Hy 3 and potato virus Y.

Experiment VII was carried out to determine if the result depended on the age of the leaves which were used as a source of infection, but in these tests the decrease in vector efficiency with increasing infection feeding time showed the typical 5 : 3 : 1 ratio and there was no difference for leaves of different ages from the same infection plant. In experiment VI A the decrease for infection feeding times was also typical. However, a fourth series of tests, experiment VIII, was carried out in the spring of 1939, also with cucumber virus 1G, and in those *M. persicae* again failed to show any decrease in vector efficiency between 2 and 15 min. of infection feeding. The same effect is seen in the results of experiment III (Table V) in which the percentage of infection with cucumber virus 1Y was nearly doubled when the infection feeding was increased from 2 to 15 min.

There is little doubt that in those experiments where this effect occurs it is not due to chance, but as it does not always occur it provides no evidence of a fundamental difference between the viruses in their response to conditions introduced by the vectors. It is more probable, as is suggested in § 10, that temporary conditions in the host plant may affect the localization of cucumber virus 1, and its availability to *M. persicae*.

*Macrosiphum gei* again seems to behave differently from the other insects with all the viruses. The decrease in infectivity of the insects was very rapid between 2 and 15 min. infection feeding, and negligible with longer infection feeding. The relation between the results for the different infection feeding times was approximately in the proportion of 6 : 1 : 1, which is in striking contrast to the "typical" ratio of 5 : 3 : 1. Taken in conjunction with the very large difference in general vector efficiency between *M. gei* and the other aphides the differences in the effects of preliminary fasting and infection feeding may be of importance, even if they were not individually significant.

(c) *Experimental conditions*

The effect of external conditions on the efficiency of the vectors and infectivity of the viruses can be observed in the variation between the weekly repetitions of each experiment. It has already been stated that the accuracy of the experiments was increased by repeating many treatments on a large number of occasions, not by testing one treatment at a time on a large number of plants. The reason for this is quite obvious from consideration of the individual weekly totals for experiments I and II which are given in Tables XV and XVI.

TABLE XV. EXPERIMENT I. INDIVIDUAL WEEKLY TOTALS FOR ALL PRELIMINARY FASTING RESULTS (SEE TABLE II)

Vectors	Viruses	No. of aphides per plant	Occasions										Total
			1	2	3	4	5	6	7	8	9	10	
<i>Myzus persicae</i>	Hy 3V	1	5	5	5	8	5	10	8	12	8	5	61
	Potato Y	1	6	5	3	5	4	5	5	8	6	2	50
	Cucumber 1G	3	3	1	0	5	4	6	1	4	4	2	30
	Total		14	11	8	18	13	21	14	24	18	9	141
<i>Myzus circumflexus</i>	Hy 3S	3	5	0	4	3	4	1	7	3	5	3	35
	Potato Y	1	1	3	1	2	3	0	4	0	1	1	16
	Cucumber 1G	3	1	1	0	2	2	4	7	2	3	3	25
	Total		7	3	5	7	9	5	18	5	9	7	76
Total			21	14	13	25	22	26	32	29	27	16	217

TABLE XVI. EXPERIMENT II. INDIVIDUAL WEEKLY TOTALS FOR ALL PRELIMINARY FASTING TIMES (SEE TABLE III)

Vectors	Viruses	No. of aphides per plant	Occasions									Total
			1	2	3	4	5	6	7	8	9	
<i>Myzus persicae</i>	Hy 3V	1	7	9	3	6	4	10	6	5	7	57
	Potato Y	1	7	6	5	4	4	8	4	6	1	45
	Cucumber 1G	3	3	7	4	7	1	5	4	3	5	39
	Total		17	22	12	17	9	23	14	14	13	141
<i>Myzus circumflexus</i>	Hy 3V	1	1	5	2	5	0	5	8	3	4	33
	Potato Y	1	1	1	0	2	2	1	4	2	1	14
	Cucumber 1G	3	6	7	7	11	5	7	7	9	8	67
	Total		8	13	9	18	7	13	19	14	13	114
Total			25	35	21	35	16	36	33	28	26	255

Although the totals indicate that *Myzus persicae* is usually the more efficient vector, on several occasions, namely occasion 7, Table XV, and occasions 4, 7 and 8, Table XVI, *M. circumflexus* did better than *M. persicae*. This interaction between occasions and the difference between *M. persicae* and *M. circumflexus* was significant in Exp. II and approached significance in Exp. I. On the other hand when *M. persicae* was compared with *Macrosiphum gei* in transmitting the same viruses, the difference in infectivity between the two aphides was more or less constant.

The most probable explanation of this result is that the two species of *Myzus* react differently to external conditions. The conditions most likely to affect the insects are those of temperature and humidity, and, as all the feedings were made in a nearly saturated atmosphere, temperature most probably accounts for the variation observed. It is not unlikely that the two insects are differently affected by temperature for the natural habitat of *Myzus persicae* is out of doors, and it is known to be adversely affected by high temperatures. *M. circumflexus* only lives in glasshouses, and probably prefers higher temperatures than *M. persicae*. *Macrosiphum gei* is also an outdoor aphid and might be expected to respond favourably to the same conditions as *Myzus persicae*, which accounts for the fact that their vector efficiencies vary between occasions in a similar manner.

#### 9. RELATIVE EFFICIENCIES OF THE APHIDES COMPARED WITH THE INFECTIVITY OF THE VIRUSES AS DETERMINED BY LOCAL LESION COUNTS

The relative quantities of virus in a solution may be determined in a variety of ways. With virus which has not been treated so as to be partially inactivated the most accurate is probably the serological method. It is generally accepted also that the infectivity of untreated sap from virus infected plants depends upon the quantity of virus which they contain, and this infectivity is generally determined either by local lesion counts, or by whole plant inoculations with varying dilutions of the sap. The close correlation between the results obtained by these three methods is strong indication that the "infectivity" measured in each case is the same thing, even though the last two methods are only empirical and are subject to large errors which cannot be controlled.

Determination of the infectivity of a group of aphides by the methods used in the present experiments is a similar process to determining the infectivity of a virus solution over a series of whole plant inoculations, because both depend on the proportion of plants which receive sufficient

virus to cause infection. However, there is no *a priori* reason for assuming that the infectivity of a group of aphides after feeding on an infected plant bears any relation to the infectivity of a sample of sap extracted from the same plant as measured by the local lesion count, whole plant inoculation or serological methods. The results already given show that the infectivity of an aphid group is not strictly comparable with that of the corresponding virus solution, for though the infectivity of the aphides undoubtedly depends to some extent on the quantity of virus available in the plant, there are many intervening factors which affect their efficiency, causing poor transmission of a very highly infective virus by such a vector as *Macrosiphum gei*, and comparatively successful transmission of a less infective virus if the vector is more efficient.

Table XVII gives average counts of the number of local lesions per half leaf for varying dilutions of the different viruses and strains, compared with each other and with Hy 3V virus. The counts were all of "starch" lesions, i.e. local concentrations of starch which are left at the points of entry of the viruses into the rubbed leaves, and which can be distinguished when the leaves are de-starched, decolorized, and stained with iodine. None of the viruses used forms necrotic local lesions which can be counted on the living leaf, except in exceptional circumstances. The arrangements used in the tests were similar to those described previously (Watson 1936). Where only two viruses were compared they were inoculated into a series of opposite half leaves, and where several were compared a randomized block arrangement of half leaves, using upper and lower leaves as "blocks" was used. Two or three sets of results are given for most of the comparisons, so as to give an idea of the variation between experiments. The last three columns in Table XVII give the mean percentage infectivity for one aphid per plant in optimum conditions of preliminary fasting and infection feeding.

Hy 3V virus seems to be the most infective according to the local lesion counts, and the most readily transmitted by all the vectors except *M. gei*. It is therefore convenient to consider the infectivity and transmissibility of the other viruses and vectors using Hy 3 virus and *Myzus persicae* as standards and Table XVIII has been constructed from the results given in Table XVII. In this table the infectivities of the viruses according to the local lesion counts have been expressed as percentages of the lesion count infectivity of Hy 3V virus, and the efficiencies of the aphides with the different viruses, as percentages of the average number of infections given by *M. persicae* with Hy 3V virus.

The infectivities of the avirulent strains of Hy 3 virus are so variable compared with the parent strain that no single estimate of their relative

TABLE XVII. COMPARISON BETWEEN THE INFECTIVITY OF THE VIRUSES AS ESTIMATED FROM LOCAL LESION COUNTS, AND THEIR TRANSMISSIBILITY BY THE DIFFERENT APHIDES

Viruses	Experi- ments*	Lesion counts per half leaf at dilutions					Mean percentage infectivity in optimum conditions when transmitted by single aphides		
		Neat	1/10	1/100	1/1000	1/10,000	<i>M. persicae</i>	<i>M. cir- cumflexus</i>	<i>M. gei</i>
Hy 3V	A	—	240	74	19	1.4	66	40	6
	B	—	—	37	13	3.1	—	—	—
	C	400	131	25	—	—	—	—	—
Hy 3W	A	1.2	—	—	—	—	2	—	—
	B	9.6	—	—	—	—	—	—	—
	C	31	3.0	1.3	—	—	—	—	—
Hy 3V	D	—	186	78	14	—	—	—	—
Hy 3S	D	—	99	33	7	—	18	15	—
Hy 3V	E	—	141	57	10	1.2	—	—	—
	F	—	227	75	34	1.6	—	—	—
Potato Y	E	—	98	21	3	0.9	39	21	10
	F	—	123	51	5	1.2	—	—	—
Hy 3V	G	—	212	—	—	—	—	—	—
	H	—	327	232	—	—	—	—	—
Cucumber 1G	G	—	120	—	—	—	20	25	2
	H	—	139	67	—	—	—	—	—
Cucumber 1Y	G	—	58	—	—	—	2	—	—
	H	—	90	44	—	—	—	—	—
Potato Y	G	—	—	—	—	—	—	—	—
	H	—	120	64	—	—	—	—	—

\* Tests carried out on opposite half leaves or in the same statistical arrangements are indicated by corresponding letters.

infectivities could be accurate. So far as Hy 3W is concerned it can only be said that both the lesion count infectivity and the transmissibility by *M. persicae* are very low, and presumably the small quantity of virus in the plants accounts for the lack of success of the vectors. The lesion count infectivity of Hy 3S virus is, on the other hand, considerably higher than its relative transmissibility by *M. persicae*. This is probably due to the intense localization of this strain in the leaf. Aphides which feed on the chlorotic patches probably have access to more virus than is necessary to make them infective, while those from the green areas may never come into contact with active virus. Hy 3S virus varies considerably in the



degree of localization of the virus in different plants, and in different leaves from the same infected plant, which accounts for the large variation between results of aphid transmission experiments.

The relative infectivity of potato virus Y according to the lesion count estimate is 60 %. Its transmissibility by *M. persicae* is 59 %, and by *M. circumflexus*, 32 %, or 52 % of the transmissibility of Hy 3V virus by *M. circumflexus*. The relative efficiency of these two vectors therefore seems

TABLE XVIII. RELATIVE INFECTIVITY OF THE DIFFERENT VIRUSES AND EFFICIENCY OF THE VECTORS EXPRESSED AS PERCENTAGES OF THE AVERAGE LESION COUNT INFECTIVITY OF HY 3V VIRUS, AND ITS TRANSMISSIBILITY BY *MYZUS PERSICAE*

Viruses	Percentage local lesion counts at dilution 1/10	Optimum percentage infectivity when transmitted by single aphidos		
		<i>M. persicae</i>	<i>M. circumflexus</i>	<i>M. gei</i>
Hy 3V	100	100	61	9
Hy 3W	1	3	—	—
Hy 3S	53	27	23	—
Potato Y	60	59	32	15
Cucumber 1G	48	30	38	3
Cucumber 1Y	27	3	—	—

to be simply related to the availability of the virus in the plant. However, *Macrosiphum gei* seems to be at least equally successful with potato virus Y as with Hy 3V virus, and it is probable that some other factor than the total quantity of virus available in the plant helps to determine the infectivity of this vector.

With the cucumber viruses there is again a more complex relationship between the efficiency of the vectors and the total quantity of virus in the plant than is shown by Hy 3V virus or potato virus Y. The relative infectivity of cucumber virus 1G according to the local lesion counts is 48 %. Its transmissibility by *Myzus persicae* is only 30 %, but its transmissibility with *M. circumflexus* is 38 %, which is 62 % of the transmissibility of this virus by *M. persicae*, and higher than would be expected from the local lesion counts. Cucumber virus 1Y is also very much less readily transmitted by *M. persicae* than would be expected from the local lesion counts.

This type of departure from a simple relationship between the efficiency of the vectors, and the total quantity of virus in the plant is associated with variation in the effect of preliminary fasting, or infection feeding

times. Thus it has been observed for *Macrosiphum gei* that the increase of vector efficiency for the first hour of preliminary fasting was greater than that shown by the other vectors, and the decrease for feeding time on the infected plants was also more rapid during the first 15 min. feeding, and did not continue after this period as it did for the other vectors. With the cucumber viruses when transmitted by *Myzus persicae*, there was no decrease in vector efficiency during the first 15 min. of infection feeding, but it can now be seen that the vector efficiency was never so high as it would have been had *M. persicae* behaved with this virus as it does with Hy 3V virus and potato virus Y. *M. circumflexus*, which showed "typical" decrease of vector efficiency with increasing infection feeding times, showed also a much higher degree of relative efficiency than *M. persicae*.

These considerations indicate that while the efficiency of the aphides as vectors does depend in part upon the infectivity of the viruses as indicated by their local lesion counts, it is also affected by other factors. These factors may be connected with the condition of the virus in the source from which it is obtained; for example, the intense localization of Hy 3S virus. They may also depend upon the physiology of the insects, as is suggested by the association of unexpected degrees of vector efficiency with variations in the effects of preliminary fasting and infection feeding times. The possible ways in which the efficiency of the aphides could be affected by such factors will be discussed later, after the nature of the preliminary fasting and infection feeding effects have been considered.

#### 10. DISCUSSION

- It has already been suggested (1938a) that the most likely explanation for the effect of preliminary fasting and infection feeding times is that the virus is inactivated by, or forms an uninfective compound with, some substance produced by the aphides. It was suggested that this substance was a secretion connected directly or indirectly with the feeding activities of the aphides, and ceased to be produced on cessation of feeding, or was produced at a much lower rate in the fasting than in the feeding insect. The experiments described in this paper have shown that the faculty is common to all the vectors studied, and to all the viruses. It seems to be possessed by the vectors in varying degrees, for the very large differences in their efficiencies as vectors cannot easily be explained in any other way.

Dykstra and Whitaker (1938) suggest that the lower efficiency of *Macrosiphum solanifolii* (probably a synonym of *M. gei*), than of other

vectors of leaf roll virus of potato, is due to the fact that *M. solanifolii* does not feed on the vascular tissues of the plants so frequently as do the other insects. This may be true of the transmission of leaf roll virus, which is persistent in the vectors, and is not transmitted by them with optimum efficiency until some time after they could have reached the vascular tissues in feeding. It could not be true of the viruses considered in this paper because the optimum period for transmission of these is less than 5 min. from the first penetration of the infected leaves. Mitchell (1937) and our own unpublished data show that none of the vectors used in these experiments can reach the vascular tissues in this time. As a rule aphides which have fed for 5 min. will only have reached the cells lying immediately below the epidermis, and therefore at the time of their optimum infectivity the virus which they transmit must have been obtained from these cells. This does not necessarily imply that the virus is more highly concentrated in these tissues. The production of inactivating substance may be so great by the time the insect reaches the vascular tissues that even though the virus is more abundant, the efficiency of the vector is decreased. It seems clear, however, that the differences in the efficiency of the vectors cannot depend on whether or not they feed from the vascular tissues.

This work on the penetration of plant tissues by the different aphides, though it is by no means complete, has also shown that there is no obvious difference in the feeding habits of the aphides which could account for the very large differences in their efficiency as vectors. It is not unreasonable, therefore, to attribute these to differences in the quantity of inactivating substance which the aphides possess, or the rate at which it is secreted. There is no direct evidence to suggest that the substance differs in quality in the different aphides, or that the viruses are differently affected by it. The relative rates of inactivation of the viruses with increased infection feeding time are similar for all the species of aphides, as also are the relative rates of increase in efficiency of the vectors with increased preliminary fasting time. It can in fact be suggested with some confidence that in its simplest form the efficiency of transmission of a virus depends on (1) the total quantity of virus present in the plant, which seems to bear, in general, a very definite relation to the amount of virus available to the aphides, and (2) the quantity of inactivating substance produced by the aphides, and the completeness with which it ceases to be produced on cessation of feeding. Thus a highly infective virus such as Hy 3V virus is more readily transmitted by all the vectors than the other less infective viruses. Also *Macrosiphum gei* which seems to have a very well developed faculty for destroying the infectivity of the viruses is an extremely bad vector of them

all. Further support to the idea that *M. gei* is a poor vector because its capacity for inactivating the viruses is greater, may be adduced from the differences in the kind of preliminary fasting and infection feeding effects obtained with it. With *M. gei* the effect of preliminary fasting was observable only for the first hour of fasting, and the decrease in infectivity with increased feeding time on the infected plant did not continue after the first 15 min. feeding. This may indicate that decrease in the production of the inactivating substance during fasting was only small, and very soon reached its limit, and that very little subsequent feeding was sufficient to restore it to its original level. When the secretion is being produced at the normal feeding rate, there appears to be an equilibrium between the quantity of virus taken in by the insect and the quantity inactivated, so that no further variation in the efficiency of the vectors is observed if the infection feeding is prolonged. There may be a similar difference between the production of the inactivating substance by *Myzus persicae* and *M. circumflexus*, but it is too small for any differences in the preliminary fasting and infection feeding effects to be apparent. ✓

There remains the problem that the aphides differ in their relative efficiencies with the different viruses. *M. circumflexus* equals *M. persicae* in its vector efficiency with cucumber virus 1G, though it is less efficient with the other viruses, and *Macrosiphum gei* is equally efficient with Hy 3V and potato virus Y, though the other vectors and the lesion counts indicate that the latter is the less infective virus. These discrepancies can be explained, as the slight disagreement between the lesion count infectivity and transmissibility of Hy 3S was explained, by the possibility that the viruses are differently localized in the tissues of the plants. As an explanation of the discrepancies in the relative efficiencies of *Myzus persicae* and *M. circumflexus*, this view is supported by the observed differences in the effect of increasing infection feeding time. In many of the experiments the maximum efficiency of *M. persicae* for transmitting the cucumber virus strains, was not reached until after the insects had fed for 15 min. on the infected plants. Also the transmissibility of the cucumber strains by *M. persicae* was less than would have been expected from their infectivities as determined by local lesion counts. It is not unreasonable to suppose that at the time of optimum vector efficiency, which for *M. persicae* is less than 5 min. from the time of penetration of the leaf, the insect has not reached the tissue in which the virus is most highly concentrated, and that by the time these tissues are reached the virus is already being inactivated by the secretion produced during feeding. If the tissue of highest virus concentration were the phloem, 15 min. would be (

the most likely time for the insects to have reached the virus infected tissues, while the rate of secretion was still below the optimum, and further decrease in their infectivity could follow. Quite a small difference in the penetration rates of the two aphides, or in the partiality of *M. circumflexus* for the virus infected tissues, would enable it to reach these tissues before its potential infectivity had been very greatly reduced. This would account for the fact that the decrease in infectivity for periods of infection feeding between 2 and 15 min. is normal for *M. circumflexus*, and that with this vector the transmissibility of cucumber virus 1 agrees with the lesion counts in being about the same as that of potato virus Y.

✓ The difference in relative efficiency of *Macrosiphum gei* with Hy 3 V virus and potato virus Y, is rather more difficult to explain. Potato virus Y may be more readily available to *M. gei* than to *Myzus persicae*, or potato virus Y may be less readily affected than the other viruses by the inactivating substance produced by the aphides, for its transmissibility by *M. circumflexus* is also slightly higher than would be expected from the local lesion counts. No information on this point can be deduced from the results so far obtained.

The results given in this paper support the hypothesis that there is a complex relationship between the non-persistent viruses and their vectors, and that one of the main factors in this relationship is the inactivation of the viruses by a substance produced by the vectors when feeding. This substance appears to be produced by all the vectors though in varying quantities. All the viruses tested seem to be affected in a similar manner, but an appearance of variability may be produced by the interaction of other factors connected with the behaviour of vectors or viruses in the host plants.

There are two possible ways in which the substance secreted by the aphides could come into contact with the virus. Either the virus is taken in by the insects and is affected by internal secretions, or the inactivating substance is ejected by the aphides with the saliva, and the virus is inactivated in the plant from which it is obtained. If the virus is inactivated in the plant then it might be transmitted by contamination of the stylets, and still be affected by the secretion of the aphides. This is unlikely for single aphides fed for only 2 min. on leaves infected with Hy 3 V virus, can infect as many as seven successive healthy plants without further access to a source of infection, so long as the feeding times on the healthy plants are reduced to a minimum. Also the decrease in infectivity of the aphides with the time taken to carry out these successive feedings, is less than when the same times were spent on only one healthy plant. The

infectivity of the aphides is in both cases very much reduced after 1 hr. of feeding on the healthy plants. Even if the aphides could carry sufficient virus on the outsides of their stylets to infect seven successive plants, more should be left after 30 min. feeding on one plant than when the aphides have penetrated the leaf five or six times during that period. Obviously the controlling factor is not the mechanical cleansing of the stylets but the quantity of inactivating substance secreted by the aphides which is probably less during the discontinuous than the continuous feedings. The details of these experiments will be published in a later paper, but reference is made to them because of the important bearing which such results must have on the very controversial question of whether or not the non-persistent viruses are mechanically transmitted. Taken in conjunction with the strong probability that the non-persistent viruses are a highly specialized group with complex virus-vector relationships, and that the highly infective tobacco mosaic group of viruses are not aphid transmissible, the fact that several healthy plants can be infected by an aphid after a single infection feeding in our opinion renders the hypothesis of mechanical infection untenable.

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#### SUMMARY

Three strains of *Hyoscyamus* virus 3, two of cucumber virus 1, and potato virus Y, were tested for their transmissibility by the aphides, *Myzus persicae*, *M. circumflexus*, and *Macrosiphum gei*. The efficiency of the vectors in transmitting all the viruses increased with increasing time of fasting before feeding on the infected plants. Their efficiency decreased as the time of feeding on the infected plants increased. The most probable explanation of these effects is that the viruses are inactivated by some substance produced by the aphides when feeding.

The most successful vector on the whole was *Myzus persicae*, and the least successful was *Macrosiphum gei*, but the relative efficiency of the vectors varied with the different viruses, indicating that their degree of success depended upon several interacting factors. The most important of these factors appeared to be:

- (a) The concentration of virus in the host plant.
- (b) The localization of virus in the host plant.
- (c) The capacity of the vector for inactivating the virus.

The viruses which have thus been shown to be similar in their insect-virus relationships, are also similar in their physical properties, and there are many other aphid transmitted viruses which resemble them in this respect. It is suggested that such viruses may form a natural group, with the same type of vector-virus relationship. This relationship appears to be a complex one, and it is unlikely that the viruses are mechanically transmitted.

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## DESCRIPTION OF PLATE 8

FIG. 1. Leaf from plant infected with Hy 3V virus, 28 days from date of inoculation. Actual length  $13\frac{1}{4}$  cm.

FIG. 2. Leaf from plant infected with Hy 3W virus, 28 days from date of inoculation. Actual length 24 cm.

FIG. 3. Leaf from plant infected with Hy 3W virus, but showing development of local Hy 3V symptoms. Two months from date of inoculation. Actual length 30 cm.

FIG. 4. Leaf from plant infected with Hy 3S virus, 25 days from date of inoculation. Actual length 21 cm.

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531.786.6:578.088.2  
611.013.3:612.015.3  
612.396.2

Morphogenesis and metabolism: studies with  
the Cartesian diver ultramicro-manometer  
V. Aerobic glycolysis measurements on the  
regions of the amphibian gastrula

BY JOSEPH NEEDHAM, VERONICA ROGERS AND SHIH-CHANG SHEN  
*From the Biochemical Laboratory, Cambridge*

(Communicated by Sir Frederick Hopkins, F.R.S.—Received 30 May 1939)

## INTRODUCTION

During the last few years various lines of work have suggested a relationship between carbohydrate metabolism and organizer phenomena (cf. the reviews of Weiss (1935), Needham (1936), and Spemann (1938)). Manometric studies of carbohydrate breakdown are therefore desirable and these became possible with the development of the Cartesian diver ultramicro-manometer. The recent investigations of Boell, Needham and Rogers (1939), using this technique, showed that in the amphibian gastrula the dorsal lip region has a higher anaerobic glycolysis than the ventral ectoderm.

The work to be described in this paper is an attempt to adapt the diver manometer to the measurement of aerobic glycolysis, and to find out





FIG. 1

A



FIG. 2



FIG. 3



FIG. 4

(Facing p. 576)



whether a similar regional difference exists in aerobic glycolysis. Owing to the technical difficulty, no previous attempts have been made to measure the  $Q\dot{O}_2$  of parts of the gastrula, although measurements of lactic acid production in air and in nitrogen of whole eggs have been made by Brachet (1934) and Lennerstrand (1933). Their results as regard aerobic glycolysis are in close agreement; in the gastrula stage a  $Q\dot{O}_2$  of about +0.05 being found. Since in both of these investigations the methods used were relatively crude, they could give no indication of regional differences.

### METHOD

The general principle of the Cartesian diver manometer has already been described (Boell *et al.* 1939). In the present experiments an adaptation, based on the "indirect method" of Warburg has been used. Pairs of divers of the same total capacity were made (20–25  $\lambda$ ), but with different liquid/gas ratios; 3:12 in one diver (*d*) and 8:12 in the other (*D*). In order that the area of the liquid/gas interface should be approximately the same in both cases, diver *D* was made with a relatively much larger bulb and shorter neck than diver *d*. It is convenient to use glass of different colours for the tails of each pair of divers.

The oxygen consumption and total carbon dioxide production can be calculated according to the original Warburg equation:

$$X_{O_2} = \frac{h k_{CO_2} - H K_{CO_2}}{\frac{k_{CO_2}}{k_{O_2}} - \frac{K_{CO_2}}{K_{O_2}}}, \quad X_{CO_2} = \frac{h k_{O_2} - H K_{O_2}}{\frac{k_{O_2}}{k_{CO_2}} - \frac{K_{O_2}}{K_{CO_2}}},$$

where  $h$  = manometric excursion of diver *d*,

$H$  = " " " " " *D*,

$k_{CO_2}$  =  $CO_2$  constant of diver *d*,

$k_{O_2}$  =  $O_2$  " " " *d*,

$K_{CO_2}$  =  $CO_2$  " " " *D*,

$K_{O_2}$  =  $O_2$  " " " *D*.

In calculating the constants of the divers, corrections have to be made for the solubilities of carbon dioxide and oxygen in oil, as described in the first paper of this series.

The reliability of the method has been shown by calibration against Warburg manometers, using yeast as material. Details of this will be given below.

## MATERIAL

Embryos of *Rana temporaria* were chosen for these experiments, early gastrulae (stage 10½–10¾) being used throughout the work. Since the two-cup method depends essentially on the two pieces of tissue being compared having the same metabolic rate, error due to this factor was reduced to a minimum by always taking lateral halves of dorsal lip and ventral ectoderm from the same egg. The dissection was carried out as usual with Spemann glass needles, and to avoid any complication of cytolysis due to imperfectly healed tissues, amphibian Ringer solution was used as a physiological medium rather than Holtfreter solution. It was found that in this medium healing was far more rapid and complete, and the tissues remained in excellent condition throughout the whole experiment. After being allowed to heal for half an hour, they were transferred to Ringer solution to which sufficient sodium bicarbonate has been added to bring it into equilibrium with 5 % carbon dioxide in the gas mixture. The subsequent procedure was exactly the same as described in the first paper of this series, except that the gas mixture used was oxygen with 5 % carbon dioxide. The total nitrogen of the tissue used was determined at the end of each experiment in the usual way by the ultramicro-Kjeldahl method of Needham and Boell (1939).

## CALIBRATION

In order to test the method, aerobic fermentation of bakers' yeast was studied, experiments being run in parallel with Warburg cups and divers. Equal amounts of yeast (*ca.* 15 mg.) were put into the cups from a 0.5 % suspension (wet weight), and made up to the appropriate volumes with 1 % glucose containing bicarbonate. In some cases, phosphate buffer and pure oxygen were used instead of bicarbonate and oxygen/carbon dioxide mixture, since yeast produces no fixed acid. For the divers, the amount of yeast used was about 1/1500 of that in the Warburg cup. The satisfactory agreement between the two methods is shown by the following results:

Exp.	$Q_{O_2}$		$Q_L^0$	
	Warburg	Diver	Warburg	Diver
T 5 (phosphate)	- 24.0	- 22.5	+ 11.0	+ 12.0
	- 20.0		+ 8.5	
T 6 (phosphate)	- 18.0	- 25.0	+ 8.5	+ 10.0
	- 25.0	- 22.5	+ 6.3	
T 9 (bicarbonate)	- 13.2	- 19.1	+ 5.0	+ 4.5
	- 14.4	- 13.9	+ 5.0	+ 3.5

There will obviously be differences between different samples since the calculations were based on wet weight. We noticed that the scattering of the  $Q_0$  and  $Q_0^L$  as determined by the two methods lies within the same limits.

In these preliminary experiments, the indirect method was also checked against direct estimation of  $Q_{O_2}$  by single divers. The use of diver technique for measuring respiration has already been described and shown to be reliable by Boell and Needham (1939). Some typical results follow:

<b>Exp. T7</b>	$Q_{0.1}$	$Q_{0.2}^0$
<b>Indirect</b>	-20.6	+13.1
<b>Direct</b>	$\begin{cases} -19.5 \\ -20.4 \end{cases}$	

## RESULTS

The data obtained from our experiments are summarized in Tables I and II, and curves from a typical experiment are given in fig. 1. In cases where an excess of carbon dioxide was found, the calculation of  $Q\dot{V}_2$  was made on the basis of an R.Q. of 1.0 for the dorsal lip and 0.85 for the ventral ectoderm. These figures were obtained by Boell, Koch and Needham (1939) using the Cartesian diver manometer.

Exp. AoGR (3)		
● $\frac{d}{D}$ } Ventral ectoderm		× $\frac{d}{D}$ } Dorsal lip region
	Dorsal lip region	Ventral ectoderm
$X_{O_2}$	-3.13λ/mg. N/hr.	-2.43λ/mg. N/hr.
$X_{CO_2}$	+2.98λ/mg. N/hr.	+2.10λ/mg. N/hr.
R.Q.	0.96	0.87

The tables show that except for a very few cases, no aerobic glycolysis can be detected either in the dorsal lip region or the ventral ectoderm. Where it exists, the amount is negligible.

The average figures for  $Q'_0$ .

**Dorsal lip** 3-74  
**Ventral ectoderm** 3-78

and the medians confirm in *Rana temporaria* what has already been found for *Discoglossus pictus* and *Amblystoma mexicanum*, that no difference exists between the oxygen consumption of the two regions. A higher r.q. of the dorsal lip than of the ventral ectoderm is also indicated: 0.92 and 0.81 respectively.

TABLE I. DORSAL LIP

Exp. no.	$X_{O_2}$	$X_{CO_2}$	R.Q.	$Q_L^{O_2}$
AeG 7	-2.39	+2.03	0.85	Nil
AeG 8	-3.05	+2.71	0.89	Nil
AeG 10	-3.55	+6.65	(1.0)	+0.291
AeGR 1	-5.25	+5.15	0.98	Nil
AeGR 2	-6.4	+6.39	0.99	Nil
AeGR 3	-3.13	+2.98	0.96	Nil
AeGR 4	-2.12	+1.18	0.56*	Nil
AeGR 9	-2.27	+2.01	0.89	Nil
AeGR 16	-4.16	+4.82	(1.0)	+0.048
AeGR 17	-3.73	+3.58	0.95	Nil
AeGR 18	-4.18	+6.11	(1.0)	+0.141
AeGR 19	-4.65	+3.94	0.85	Nil
Av.	-3.74		Av. 0.92	

N.B.  $X_{O_2}$  in the table corresponds to  $Q'_{O_2}$ , i.e. oxygen consumption/mg. total nitrogen of tissue per hour. Factors for converting total nitrogen to dry weight were determined experimentally by Boell *et al.* (1939) on the same species; they are 13.7 for the dorsal lip and 15.1 for ventral ectoderm. In this way  $Q_L^{O_2}$  above is calculated. The respiratory quotients shown in brackets are those assumed as described in the text.

\* Excluded from average.

TABLE II. VENTRAL ECTODERM

Exp. no.	$X_{O_2}$	$X_{CO_2}$	R.Q.	$Q_L^{O_2}$
AeG 7	-3.41	+2.83	0.83	Nil
AeG 8	-2.86	+2.80	0.98	Nil
AeG 9	-1.81	+2.06	(0.85)	+0.034
AeG 10	-2.6	+1.30	0.52	Nil
AeGR 2	-4.65	+4.36	0.94	Nil
AeGR 3	-2.43	+2.1	0.87	Nil
AeGR 4	-1.41	+0.47	0.34*	Nil
AeGR 9	-6.52	+6.88	(0.85)	+0.09
AeGR 16	-5.3	+4.5	0.85	Nil
AeGR 17	-4.01	+4.28	(0.85)	+0.059
AeGR 18	-5.49	+4.35	0.80	Nil
AeGR 19	-4.6	+6.2	(0.85)	+0.147
AeGR 20	-3.95	+2.87	0.72	Nil
Av.	-3.78		Av. 0.81	

N.B. Symbols as in the previous table.

\* Excluded from average.

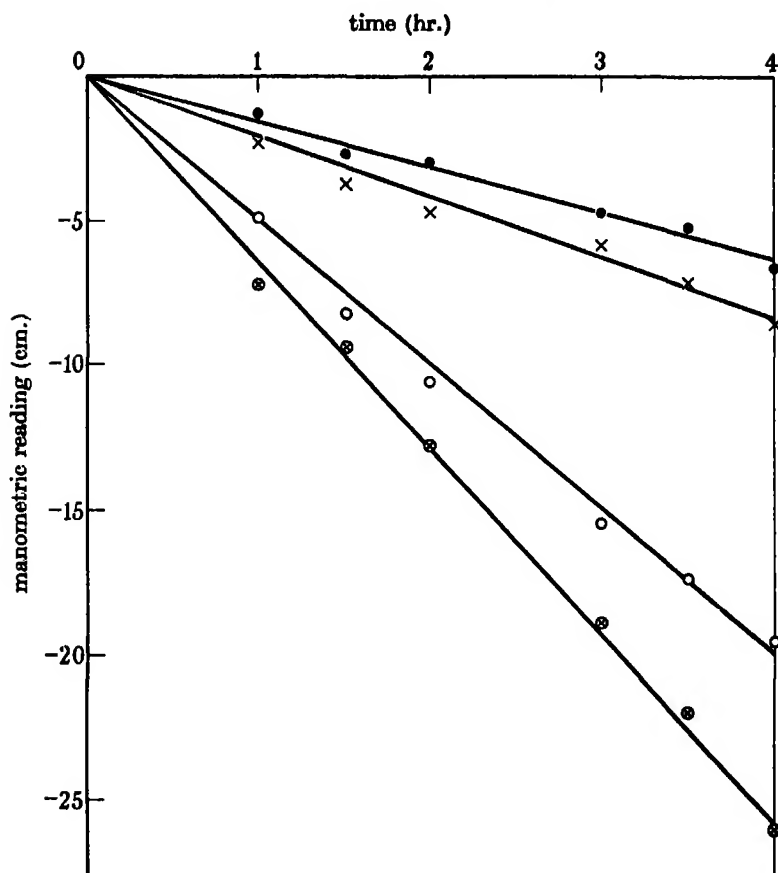


FIG. 1

## DISCUSSION

As recognized by Dixon (1934), the two-cup method of Warburg is not as accurate as other manometric procedures owing to the impossibility of ensuring that the two pieces of tissue, on the comparison between which the method depends, are in fact metabolizing in exactly the same way and at the same rate. It is, nevertheless, indispensable for the measurement of aerobic glycolysis unless one of the high-pressure methods (Dickens and Šimer 1931; Dixon and Keilin 1933) is employed. Up to the present time the diver technique has not been adapted for such measurements, though we do not believe that this would present insuperable difficulty.

The results reported in this paper show fairly conclusively that the aerobic glycolysis of the amphibian gastrula, if it exists, is exceedingly

small. Substantial values were obtained only in exceptional cases (three experiments out of twenty-five) and in four further experiments there were minute excess carbon dioxide outputs corresponding roughly to the figures of Brachet (1934) and Lennerstrand (1933) obtained by lactic acid analysis on large quantities of embryos. In general there is no measurable lactic acid production in aerobic conditions, and most important of all, there is no difference in behaviour between the organization centre and the inert ventral ectoderm.

In the foregoing calculations no allowance has been made for the aerobic ammonia production to be expected. As previously noted one amphibian gastrula produces approximately  $1\mu\text{g. ammonia/mg. dry wt./24 hr.}$  (Białasiewicz and Mincówna 1921; Boell *et al.* 1939).  $100\mu\text{g. dry wt. tissue}$  (the amount usually in the diver manometers) would therefore be expected to produce  $0.0041\mu\text{g./hr.}$ ; a quantity corresponding to  $0.0039\lambda$  carbon dioxide/hr. or  $0.5\lambda/\text{hr./mg. total nitrogen}$ . This masked excess carbon dioxide output would mean a very low  $Q\dot{O}_2$  of the order of  $+0.03$ , but as ammonia estimations were not regularly made, such figures are not incorporated in the tables.

The significance of aerobic glycolysis is still by no means fully elucidated, but in most tissues except tumours it is small, since the Pasteur effect is usually relatively efficient. In this the embryos of birds and amphibia agree. The classical picture of embryonic metabolism as showing a high anaerobic glycolysis, a high respiratory rate, and no aerobic glycolysis, is not quite correct, as  $4\frac{1}{2}$ -day chick embryos give a  $Q\dot{O}_2$  of about  $+1.0$  (Needham, Nowiński, Dixon and Cook 1937). Laser (1937*a*) went further in showing that in earlier stages, e.g.  $2\frac{1}{2}$  days' incubation, when  $Q_{O_2}$  is as high as  $-25.4$ ,  $Q\dot{O}_2$  may be as high as  $+10.8$ . Laser's evidence suggested that the Pasteur effect passes through a maximum of efficiency at 91 hr. incubation, for at 7 days  $Q\dot{O}_2$  was up to  $+5.8$  again (slices) though the  $Q\dot{O}_2$  is falling slightly, not rising.

This goes far to dispose of the belief, fairly widespread in the literature, that aerobic glycolysis is in some way connected with unspecific injury to the tissue. Negelein's original conclusions on rat embryos in serum and in Ringer solution (1925) are not borne out by his own figures. Laser (1937*b*) has shown, moreover, that in conditions of low oxygen tension (5–20% oxygen) which no doubt reproduce the physiological tension more closely than that of the gas mixtures generally used (95% oxygen), aerobic glycolysis is considerably increased. Chick embryo allantois, for example, showed a zero  $Q\dot{O}_2$  in 95% oxygen but  $+13.6$  in 5% oxygen. It would be interesting to repeat the experiments in this paper using lower oxygen concentrations.



The main point of importance in the present paper is that whatever may be the characteristics of metabolism inside and outside the organization centre, the Pasteur effect seems equally efficient in all regions investigated.

## SUMMARY

1. The Cartesian diver manometer has been adapted for the measurement of aerobic glycolysis by the two-cup method.

2. Aerobic glycolysis is exceedingly small or absent in both dorsal lip region and ventral ectoderm of *Rana gastrulae*. Hence the two regions do not differ as regards the efficiency of the Pasteur effect.

3. The absence of any difference in respiratory rate between these two regions, previously observed in gastrulae of *Discoglossus* and *Amblystoma*, has been confirmed for *Rana*.

4. That there is a difference in respiratory quotient between the two regions during gastrulation, previously observed on *Amblystoma*, has been verified on *Rana*.

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## ERRATA

*Paper by E. C. Dodds, L. Golberg, W. Lawson and Sir Robert Robinson*

*p.* 140. The bibliographic reference number should read

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*Paper by A. Haddow and A. M. Robinson*

*p.* 277. The bibliographic reference number should read

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616-066-46-02:547.68

*Paper by J. Ferguson*

*p.* 396. Line 10 from bottom, *delete* 10<sup>-6</sup>

*pp.* 403, 404. In the references to Frunkin and to Schilow and Nekrassow  
for *Z. physik.* read *Z. phys. Chem.*

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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3 FEBRUARY 1939

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**The use of chemical potentials as indices of toxicity.** By J. FERGUSON.  
(Communicated by A. J. Clark, F.R.S.—Received 1 December 1938.)

From a review of published data it is concluded that two main classes of toxic action exist. In one class the effects are due to chemical reaction. In the other a physical mechanism is at work. Criteria are given whereby the two classes of toxic action may be distinguished. All substances can exert physiological effects by a physical mechanism though the physical action may be, and frequently is, masked by a specific chemical reaction.

Physiological effects due to a physical mechanism are generally measured when an equilibrium has been established between the concentration of the toxic substance in the phase, solution or vapour, in which it is applied and the concentration in the phase or surface layer which is the seat of toxic action. Since the chemical potential of the toxic substance must be the same in all phases partaking in the equilibrium, it is suggested that the toxicities of physically toxic substances should be compared, not by the values of the toxic concentration in the external solution or vapour, but by the values of the chemical potentials in those phases. Chemical potentials so determined are identical with the chemical potentials at the actual point of attack within the organism. By this procedure the disturbing effect of phase distribution is eliminated from the comparison of toxicities.

Using the activity function of G. N. Lewis as the chemical potential it is found that, when chemical reaction is absent, though diverse chemical compounds exert the same toxic effect on a given organism at widely different concentrations, the activities corresponding to these concentrations lie within a relatively narrow range. The differences in activity within this range are ascribed to the effect of chemical constitution.

**The effect of X-rays on the glucose and hexose phosphate glycolysis of tumour tissue.** By B. E. HOLMES. (*Communicated by Sir Frederick Hopkins, O.M., F.R.S.—Received 12 December 1938.*)

Recent work describing the chemical effects of X- and  $\gamma$ -irradiation on substances, in solution, and the effects of X- and  $\gamma$ -irradiation upon the metabolism of cells is described and discussed.

It is shown that a dose of X-radiation sufficient to cause a 50–100 % inhibition of lactic acid formation from glucose by tumour tissue, has no effect on lactic acid formation from hexose-diphosphate, hexose-monophosphate (Emden ester) or glucose-*L*-phosphate (Cori ester).

Results are given showing the effect of  $\gamma$ -radiation on the lactic dehydrogenase system of young embryo brains.

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**The fluorescence of compounds containing manganese.** By J. T. RANDALL. (*Communicated by M. L. E. Oliphant, F.R.S.—Received 9 December 1938.*)

One of the most important problems to be solved in the field of fluorescent solids is the role played by impurities or "activators", of which manganese is one of the best known. In the present paper the fluorescence of more than twenty compounds containing measured traces of this element has been studied at low temperatures. The interpretation of the results has been helped considerably by the discovery of red fluorescence bands for the pure halides of manganese, and in the first part of the paper results for these are given. The evidence obtained suggests that the fluorescence of the manganous halides is characteristic of all the manganese atoms in the crystals, and that the transitions concerned are of the forbidden type (probably  ${}^2G-{}^4F$ ) lying within the divalent ion  $Mn^{++}$ .

In the second part of the paper the fluorescence of solids containing manganese impurity has been examined at low temperatures. Four main classes have been found: (a) solids in which the manganese impurity is entirely responsible for the fluorescence spectrum, which corresponds very closely to that of the pure halides of manganese; (b) fluorescence also due to the manganese impurity, but the surrounding ions have an influence on the position of the spectrum differing from that of class (a); (c) due both to the matrix lattice and to the incorporated manganese; (d) more complex cases which are recognizable as examples of multiple impurities. Cases intermediate to these have also been found.

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**The measurement of magnetic saturation intensities at different temperatures.** By W. SUCKSMITH. (*Communicated by A. M. Tyndall, F.R.S.—Received 12 December 1938.*)

A new method of measuring the saturation intensities of ferromagnetic substances at all temperatures up to the Curie temperature is described. The pole-pieces of an electromagnet are shaped to produce a slight gradient in the field in a direction



perpendicular to the lines of magnetic force. The specimen is thus acted upon by a force which is directly proportional to the product of the mass and the saturation intensity. The small movement of the specimen is limited to a region where the field gradient is uniform. Suitable magnification ensures that the relationship between magnetic moment and deflexion is linear to a high degree of accuracy. The force is of the order of a few grams weight, though only a few cubic millimetres of substance are required.

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**Researches on plant respiration. V. On the respiration of some storage organs in different oxygen concentrations.** By J. K. CHOUDHURY. (*Communicated by W. Stiles, F.R.S.—Received 13 December 1938.*)

The course of respiration of potato and artichoke tubers and of carrot roots, in a wide range of oxygen concentrations between zero and 98.3 %, was followed continuously for periods of several days at 25° C. The course of respiration of red beet roots in air and nitrogen was also examined.

In all the tissues employed the course of respiration exhibited two phases, the respiration intensity first rising to a maximum and then falling gradually, sometimes to a constant value. The initial rise in respiration rate may be largely due to the evolution of dissolved carbon dioxide from the tissues as a result of the rise in temperature which occurs when the tissues are transferred to the experimental conditions.

There was some evidence of a seasonal drift in the respiratory activity of both potatoes and artichokes.

After the tissues had been subjected to the experimental conditions for 100 hr. in air, the average rate of carbon dioxide production of the different tissues examined was 0.46, 2.00, 1.57 and 1.26 mg. per 100 g. of tissue per hour in potatoes, carrots, artichokes and red beet roots respectively.

Varying oxygen concentration between 6.2 and 98.3 % inclusive did not affect the respiratory activity of potatoes. With artichokes, however, this was only the case with concentrations ranging from that of air upwards. With concentrations of oxygen below 20 % the respiration rate altered with every alteration in the concentration of oxygen. The respiration rate of carrots was affected by the concentration of oxygen throughout the whole range of oxygen concentrations employed. In the lowest concentration of oxygen used, 3.5 %, the respiration intensity was in part determined by the size of the carrots.

In an atmosphere of nitrogen the respiration intensity of potatoes, artichokes and red beets was lower than in air, but on transferring carrots from air to nitrogen the respiration intensity gradually rose to a higher level, the increased rate being maintained even for 117 hr.

In potatoes transference from anaerobic to aerobic conditions, even with only 6.2 % oxygen, brought about a rapid rise in the respiration rate to a value considerably above the normal rate in air, this being followed by a fall to the normal rate in air, but the amount of carbon dioxide given out in air before the resumption of the normal rate did not always show a direct relation with the length of the period of anaerobiosis.

A consideration of the experimental results suggests that oxygen is not essential for the production of the actual substrate for glycolysis, nor does it seem to have any direct control over the rate of production of the substrate.

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**Self-consistent field, including exchange and superposition of configurations, with some results for oxygen.** By D. R. HARTREE, F.R.S., W. HARTREE and B. SWIRLES. (*Received 13 December 1938.*)

In the simplest approximation to the structure of a many-electron atom, each term is regarded as arising from a single configuration of one-electron wave functions. When, to this approximation, two or more configurations give terms of the same parity,  $L$ , and  $S$ , not greatly different in energy, an appreciably better approximation may be obtained by taking, as the wave function for each term, a linear combination of the wave functions for the various configurations. The term "superposition of configurations" is used to denote the use of such linear combinations.

The effect of this superposition of configurations can be taken into account in the calculation of energy values only, or in the determination of the one-electron wave functions also. The latter requires an extension of Fock's equations, and in the present paper this extension is worked out for the superposition of the  $(np)^{s+2}$  configuration on the normal  $(ns)^2 (np)^s$  configuration of atoms with an incomplete 8-shell. The solution of these equations involves a set of calculations, each of which is similar to a complete solution of Fock's equations, for a set of trial values of a parameter expressing the relative amplitudes of the wave functions for the two configurations.

For  $O^+$ , the normal configuration  $(2s)^2 (2p)^3$  gives  $^4S$ ,  $^4D$  and  $^4P$  terms, of which only the highest ( $^4P$ ) is subject to the superposition of the  $(2p)^4$  configuration. A full solution of the extended equations has been worked out for this case. The effect of superposition of configurations on the radial wave function is found to be small, less than 1 in 200 of their greatest values, and the consequent effect on the total energy of the  $(2s)(2p)$  shell very small, about 1 in 4000. This small improvement of the energy value did not seem to justify the solution of the extended equation for other states of ionization, and for these solutions of the ordinary Fock equations only have been evaluated, though superposition of configurations has been taken into account in calculating energy values.

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**The place of the megaloblast in the development of red blood cells.** By M. C. G. ISRAELS. (*Communicated by H. S. Raper, F.R.S.—Received 14 December 1938.*)

The morphology of the erythroblastic cells obtained by biopsy from human bone-marrow in hyperplastic conditions of the erythropoietic tissue has been studied. The types of cells appearing have been defined.

True megaloblasts only occur when the hyperplasia is the result of lack or imperfect working of the anti-pernicious-anaemia liver principle. In all other conditions cells

of the normoblast series are present. There are also certain primitive cells usually present in *all* types of hyperplasia, the pro-erythroblasts and haemocytoblasts.

The normal development of the red blood cells probably proceeds from haemocytoblast to pro-erythroblast, and then to normoblasts of varying degree of maturity. The megaloblasts also develop from the pro-erythroblasts and then follow their own line of development to mature as red blood cells.

The megaloblasts have no place in the development of normal red blood cells in extra-uterine life; they appear only when the proper activity of the liver principle is in abeyance. So long as the liver principle is normally available, demands for increased red blood cells, whatever their cause, are met by proliferation of cells of the normoblastic series.

It is tentatively suggested that the megaloblasts represent an early phylogenetic type of red blood cell which is a transient phase very early in the growth of the human embryo, and that they possibly do not require the liver principle to enable them to mature completely. Their appearance in pathological disturbances of the liver principle's functions is possibly of the nature of an attempted compensation for the loss of normoblastically developed red cells.

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**Transfer effect in liquid He II. Part I. The transfer phenomena.** By J. G. DAUNT and K. MENDELSSOHN. (*Communicated by F. A. Lindemann, F.R.S.—Received 14 December 1938.*)

In two co-axial vessels filled to different levels with liquid He II, helium is transported from the higher to the lower level until both levels have the same height. This "transfer" takes place in a surface film of helium covering all solid surfaces in contact with the liquid. The rate at which the transfer takes place is practically independent of the difference in height between the levels. The transferred amount is exactly proportional to the width of the connecting surface and is the same for glass and polished copper. If a beaker containing liquid He II is lifted out of the helium bath the transfer takes place although there is no solid surface connecting beaker and bath. The liquid runs over the rim of the beaker and collects in drops at the bottom and drips into the bath until the beaker is empty. The transfer from one container with liquid He II to another is limited by the narrowest part of the connecting surface *above* the higher level. Constrictions below the height of the upper level do not limit the flow as at such places helium can drop off as free liquid.

Although the transfer takes place from a higher to a lower level, the independence of the flow on the difference in level shows that gravity is not the driving force.

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**The transfer effect in liquid He II. Part II. Properties of the transfer film.** By J. G. DAUNT and K. MENDELSSOHN. (*Communicated by F. A. Lindemann, F.R.S.—Received 14 December 1938.*)

The properties of the surface film in which the transfer of He II takes place are investigated. In view of the exceedingly high heat conductivity of the bulk liquid

it was thought possible that the helium film might also show high heat conduction. The experiments, however, yield the surprising result that the actual heat "conductivity" is poor and that the high heat transport is due to a constant flow of matter through the film from the cold to the warm place.

The thickness of the transfer film was found to be about  $3.5 \times 10^{-8}$  cm., i.e. 100 atoms thick. No appreciable change in thickness was observed between the  $\lambda$  point and  $1.5^\circ$  K. Above the  $\lambda$  point the film is not more than  $10^{-7}$  cm. thick.

The rate of transfer per unit surface changes with temperature. It is zero at the  $\lambda$  point and increases with falling temperature to  $7.5 \times 10^{-5}$  c.c. of helium per second at  $1.5^\circ$  K. Below  $1.5^\circ$  K it is practically independent of temperature. In conjunction with our determination of the film thickness we thus arrive at an average velocity of 20 cm./sec. for the transfer.

None of the theories of liquid He II so far gives an indication why the transfer film exists or why the transfer takes place. Our experiments have led us to the assumption that a transfer takes place when from a solid surface which is in contact with liquid He II, the film is removed. Then the bared surface is readily covered again with helium from the liquid at a rate which solely depends on the thermal state of liquid He II. Our experiments lead also to the suggestion that the high heat conductivity of the free liquid is a transport phenomenon of a similar kind.

### **Nitrogen, argon and neon in the earth's crust, with applications to cosmology.** By LORD RAYLEIGH, F.R.S. (*Received 15 December 1938.*)

It is found that plutonic rocks contain nitrogen, argon and neon. The nitrogen appears to be mainly in chemical combination. The argon and neon are probably trapped in crystal lattices.

The argon found usually amounts to about  $2 \times 10^{-4}$  c.c./g. The neon is about  $8 \times 10^{-8}$  c.c./g.

The ratio neon/argon is of the same order of magnitude as the ratio of those gases in the atmosphere, and does not much favour the view that there has been a loss of neon from the atmosphere.

The vesicles of pumice stone contain an altogether exceptional proportion of neon. This is difficult to understand, but can scarcely be considered to affect the general problem.

Rocks in general contain about 0.04 c.c. of nitrogen per g. It has hitherto been supposed that terrestrial nitrogen is mainly concentrated in the atmosphere, but on the basis of the present results, the rocks probably contain in all some fifty times as much nitrogen as the atmosphere. It is no longer necessary therefore to regard the atmosphere as primitive, and no special difficulty arises as to why the earth did not lose its (dissociated) nitrogen when the temperature was high. It may have lost some, but there was more in the interior.

**The analysis of discrete vector maps.** By D. M. WRINCH. (*Communicated by I. Langmuir, For.Mem.R.S.—Received 16 December 1938.*)

The study of crystals by X-rays, while permitting the observation of the amplitude of the structure factor for various planes, does not determine the phase. Exhibited in the form of structure factors, the data obtained by X-ray analysis necessarily involve a multiple infinite set of unknown and unobservable parameters. The vector map of the crystal, however, depending only on the amplitudes and not on the phases, gives a complete picture of all the information available by this technique. The vector map of a crystal comprises a discrete set of point intensities. The crystal may then be regarded as a uniform distribution of electrons upon which is superposed a set of discrete electron density deviations or "diserons". Any set of diserons in atomic space  $S_1$  gives a corresponding set of diserons in vector space  $S_2$ . To discover the structure of the crystal from X-ray observations, it is therefore necessary and sufficient to calculate the vector function and then to find all the  $S_1$  diseron sets corresponding to the given  $S_2$  diseron set. In this way vector maps can be used to discover crystal structures as well as to confirm or refute structures already proposed.

In practice, an experimentally obtained vector map may, by a process of successive approximation, be regarded as a series of simple point sets of gradually increasing complexity. The present communication deals with the structure of various simple finite point sets and investigates analytically the  $S_2$  sets corresponding, special attention being paid to sets which are trigonal and hexadic. The transition between finite and periodic  $S_1$  sets and their corresponding  $S_2$  sets is accomplished. In general an  $S_2$  point set uniquely determines a corresponding  $S_1$  point set. In degenerate cases, however, there is a certain severely limited multiplicity of  $S_1$  point sets. In this case also the crystal structure and indeed the molecular structure can be systematically investigated by successive approximation. As an illustration, a certain  $S_2$  set, representing to a first approximation a vector diagram of insulin, is considered and the deductions regarding the structure of insulin are given.

**Sums of positive integral powers.** By H. DAVENPORT. (*Communicated by L. J. Mordell, F.R.S.—Received 16 December 1938.*)

Let  $N_s^{(k)}(n)$  denote the number of integers less than  $n$  that are representable as the sum of  $s$  positive integral  $k$ th powers. A method is given for improving on the previously known lower bound for  $N_s^{(k)}(n)$ . In particular, it is proved that, for any  $\epsilon > 0$ ,  $N_s^{(k)}(n) > n^{\alpha(s,k)} \epsilon$ , for all large  $n$ , where

$$\alpha(3, 3) = \frac{1}{18}, \quad \alpha(3, 4) = \frac{2}{3}, \quad \alpha(4, 4) = \frac{1}{2}, \quad \alpha(5, 4) = \frac{7}{8} + \frac{1}{2} \epsilon.$$

These results allow one to prove that

- (1) almost all numbers are the sum of four positive cubes;
- (2) every large number which does not leave the remainder 15 or 16 on division by 16 is the sum of 14 fourth powers.

In particular, every large number is the sum of 16 fourth powers. It was already known that no lesser number than 16 is enough.

**Critical and co-operative phenomena. IV. A theory of disorder in solids and liquids and the process of melting.** By J. E. LENNARD-JONES, F.R.S. and A. F. DEVONSHIRE. (*Received 20 December 1938.*)

A theory of the process of melting of a pure solid of one component is worked out. It is based on the hypothesis that the essential difference between a solid and a liquid is that one is ordered and the other disordered and that a change from one state to the other can be followed by a continuous transition of a variable suitably chosen to represent the state of order. This theory is alternative to that given in a former paper, but it leads to similar results. Formulae are given for the melting temperature, the volume change on melting and the coefficient of expansion of liquids in terms of interatomic forces.

Lindemann's formula for melting is found to be a special consequence of the theory and it is interpreted in terms of the force fields of atoms.

The theory indicates the existence of a critical temperature above which there would not be a sharp transition from solid to liquid.

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**Interaction between adsorbed substances of simple constitution and insoluble monolayers.** By N. K. ADAM, F.R.S., F. A. ASKEW and K. G. A. PANKHURST. (*Received 30 December 1938.*)

The interaction between simple capillary active solutions and insoluble monolayers has been studied by measuring the surface pressure and surface potential of the insoluble monolayers, put on the surface of the solutions. Equilibrium is established practically instantly. Penetration generally, but not invariably, occurs at surface pressures of the order a few tenths of a dyne per centimetre, phenol having, as a rule, the greatest penetrating power, butyric acid, the next, and butyl alcohol the least, of these three capillary active solutes. The principal result of penetration is to disrupt the cohesion of the insoluble film, turning a normally coherent film into a vapour expanded or gaseous one. Penetration may continue up to much higher surface pressures, but more frequently, when the film is compressed, penetration diminishes or ceases altogether, and the adsorbed is then usually displaced more or less completely from the surface by the insoluble film. No evidence has been found of stoichiometric complex formation between the adsorbed and the insoluble molecules, in these cases; and the evidence of specific interaction between the active groups in the insoluble and the adsorbed film is in these cases very scanty.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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7 MARCH, 1939

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**Structure and thermal properties associated with some hydrogen bonds in crystals. Part II. Thermal expansion.** By J. M. ROBERTSON and A. R. UBBELOHDE. (*Communicated by Sir William Bragg, O.M., P.R.S.—Received 12 December 1938.*)

The total thermal expansion of NaCl (rock salt), oxalic acid dihydrate  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  and  $(\text{COOD})_2 \cdot 2\text{D}_2\text{O}$ , and resorcinol  $\text{C}_6\text{H}_4(\text{OH})_2$  and  $\text{C}_6\text{H}_4(\text{OD})_2$  have been measured by X-ray spectroscopy, over the temperature interval 90–200° K. Polar diagrams have been calculated giving the proportional expansion in various directions in the crystal. Marked anisotropy of thermal expansion is observed in crystals containing hydroxyl and hydrogen bonds. The expansion of the deuterium crystals is slightly smaller than that of the hydrogen crystals. Possible interpretations of these observations are discussed in terms of the role of hydrogen and hydroxyl bonds in the crystal structure.

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**The histology and self-differentiating capacity of the abnormal cartilage in a new lethal mutation in the rat.** By H. B. FELL and H. GRÜNEBERG. (*Communicated by J. B. S. Haldane, F.R.S.—Received 28 December 1938.*)

The two chief abnormalities which characterize the cartilage of the lethal rats are (1) the formation of thick capsules around the chondroblasts and (2) very active proliferation and chondrogenesis in the perichondrium. In rats of less than 16 hr. these abnormalities have only just begun to appear. The abnormalities do not develop to the same degree or at the same time in all parts of the cartilaginous skeleton.

When cartilage from young lethal rats is explanted *in vitro* the cartilage survives in a healthy condition for about 12 days, but does not grow, and the abnormality progresses little if at all during cultivation. Normal costal cartilage when implanted subcutaneously in young lethal rats grows well but remains normal. Cartilage from young rats when implanted subcutaneously into normal rats grows vigorously and continues to develop its characteristic abnormality. Given sufficient time, the transplanted cartilage develops the anomalies to an extent never reached during the short life-span of a lethal.

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**The general motion of the aeroplane.** By S. BRODETSKY. (*Communicated by L. Bairstow, F.R.S.—Received 30 December 1938.*)

The only information concerning general aeroplane dynamics, unassociated with steady motion and small disturbances, is the theory of Lanchester's phugoids, published in 1908. There has thus been a generation of stagnation in this regard, and the object of the paper is to initiate a systematic study of the subject.

The general idea is to deduce, in the first instance, an approximate solution, and then improve it, if necessary, by proceeding to higher approximations. The mathematical process consists in discarding the time as the independent variable, and using one of the Eulerian angles instead. We assume that one component of velocity of the centre of gravity predominates over the other two components, and that the angular motion is slow. Using experimental evidence as to the orders of magnitude of the various terms in the equations of motion, we find a large number of cases in which the first approximations admit of comparatively easy solution.

The method applies to all ordinary aeroplanes, with or without engine; also to moving controls, as in the use of the elevator when flattening out from a dive.

We find that Lanchester's phugoids represent a first approximation to general longitudinal flight, when the machine is in normal condition, but in circumstances not usually realized in practice. New types of first approximation paths are found, referring to circumstances that are realized in practice. The method also gives "three-dimensional phugoids" that suggest the Immelmann turn, and other new three-dimensional paths, as well as the slow spin of the stalled aeroplane, and the slow roll.

First approximations to important aerobatics have thus been obtained; some have been worked out in detail already, while others are now being investigated by the writer and collaborators. Second approximations have been obtained for looping, and for rolling motion.

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**Infra-red analysis applied to the exchange reaction between ethylene and deuterioethylene.** By G. K. T. CONN and G. H. TWIGG. (*Communicated by E. K. Rideal, F.R.S.—Received 3 January 1939.*)

The interaction of ethylene ( $C_2H_4$ ) and deuterioethylene ( $C_2D_4$ ) on a nickel catalyst has been examined with a view to finding out whether any exchange occurs between them under conditions where exchange between ethylene and deuterium takes place.



Analysis was carried out by observing under high dispersion the infra-red absorption spectra of the  $C_2H_4-C_2D_4$  mixture from  $6.9$  to  $9.3\mu$  before and after heating on the catalyst. In particular the  $Q$  branches of the  $C_2H_4$  and  $C_2D_4$  deformation frequencies at  $6.92$  and  $9.25\mu$  respectively were carefully examined to find out whether any diminution in the  $C_2H_4$  or  $C_2D_4$  pressures had occurred. The region between these two frequencies was investigated for evidence of the "cross compounds" which would arise from exchange.

The absorption spectra of the mixture before and after heating were materially identical and so exchange between the two ethylenes could be detected, within the limits imposed by the sensitivity of the technique.

The sensitivity of the method of detection of exchange was investigated using the  $C_2H_4$  and  $C_2D_4$   $Q$  branch absorptions. A diminution in the ethylene pressure corresponding to one-sixth of complete reaction could have readily been detected.

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**The exchange reaction between ethylene and deuterium on a nickel catalyst.** By G. H. TWIGG and E. K. RIDEAL, F.R.S. (*Received 3 January 1939.*)

The exchange and hydrogenation reactions between ethylene and deuterium on a nickel catalyst have been investigated over the temperature range  $60-207^\circ C$ . The kinetics of both reactions at  $156^\circ C$  are identical, the rate in both cases being independent of the ethylene pressure and directly proportional to the deuterium pressure. The hydrogen produced in the exchange reaction is not at equilibrium with itself. The reaction  $H_2 + D_2 \rightleftharpoons 2HD$  on the catalyst is completely inhibited up to  $160^\circ C$  owing to the strong adsorption of the ethylene, and equilibration of the hydrogen only takes place through the exchange reaction. At about  $160^\circ C$  desorption of the ethylene sets in. From a consideration of the state of equilibrium of the hydrogen at the beginning of the reaction, it is shown that each deuterium molecule entering reaction undergoes a number of exchanges before desorption; the actual exchange process is fast and the rate determining step in the reaction is the adsorption of the deuterium.

The actual exchange process takes place through the primary addition of a deuterium atom to a chemisorbed ethylene molecule forming an ethyl radical which then decomposes to give a deuterated ethylene molecule and release a hydrogen atom. On the basis of this additive mechanism, predictions have been made as to the double bond migration and the exchange reactions in higher olefines. A mechanism has been advanced to explain the course of the exchange reaction including the adsorption of the deuterium. The present experiments also suggest that in hydrogenation the hydrogen molecule adds as a whole to the ethylene and not by atoms.

The energy of activation for exchange is constant at  $18.6$  kcal. up to  $100^\circ C$ , but, above  $100^\circ C$ , becomes temperature dependent, decreasing to  $4$  kcal. at  $207^\circ C$ . The difference in energies of activation for exchange and hydrogenation is constant over the whole temperature range, that for exchange being  $4-5$  kcal. greater than that for hydrogenation. The decrease in energy of activation with increasing temperature in both reactions is attributed to desorption of the hydrogen held above the chemisorbed ethylene layer.

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**The thermal and electrical resistance of bismuth single crystals: the effects of temperature and magnetic fields.** By G. W. C. KAYE. (*Communicated by C. G. Darwin, F.R.S.—Received 3 January 1939.*)

The thermal and electrical resistivities of bismuth single crystals have been studied at a series of temperatures from 25° up to 160° C. In either case, the values for a direction parallel to the trigonal axis are greater than for those at right angles. The temperature coefficient is positive in every case, being roughly twice as large for the electrical resistivity as for the thermal.

The effects produced on the resistivities of bismuth crystals by magnetic fields up to about 20,000 gauss have been measured for various crystal orientations using fields both parallel and at right angles to the thermal or electrical flow. In all circumstances, the resistivity is increased by an amount which is invariably greater (usually substantially) for the electrical resistivity than for the thermal. The effects are most pronounced when the thermal or electrical flow is parallel to the trigonal axis, and the field is at right angles to one of the three "lines" or secondary axes. The effects are least pronounced when the field, the flow and the trigonal axis are all parallel. In all the orientations, the effects are relatively small and approximately parabolic for fields up to about 2000 gauss, thereafter becoming linear and more pronounced for stronger fields, except for one or two orientations where there is a tendency to "saturation".

The observations lend support to the view that there are two conducting mechanisms through the medium of which a magnetic field can affect the conductivity, one predominating for electrical conduction while both appear to be operative for thermal conduction, their relative contributions varying with the orientation.

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**Molecular layers (Pilgrim Trust Lecture, 1938).** By I. LANGMUIR, *For.Mem.R.S.* (*Received 5 January 1939.*)

The forces between molecules of organic liquids of non-ionic type are ordinarily of such short range that they act only when the molecules are in contact. The magnitude of the force depends mainly upon the area of and the nature of the two contacting molecular surfaces. This principle of independent surface action has been a useful guide in the development of theories of surface tension phenomena, and should be equally valuable for theories of vapour pressures, and solubilities. It leads directly to the concept of molecules having hydrophobic and hydrophilic parts which spread as oriented monolayers on a water surface.

Such monolayers can have the properties of two-dimensional gases, liquids or solids. A type of film called a duplex film, having no three-dimensional analog, has two interfaces (an upper and a lower) which are separated by a thin three-dimensional layer (the interstratum).

Expanded films, such as monolayers of myristic acid on acidulated water, are duplex films in which the interstratum is a hydrocarbon liquid. The lower interface, contains all the hydrophilic groups. These, because of thermal agitation, constitute a two-dimensional gas that exerts the surface pressure causing the expansion of the film.

Many proteins, although very soluble in water, form remarkably insoluble monolayers which are duplex films. The interstratum consists of polypeptide chains which form loose loops attached at intervals to the upper interface by hydrophobic groups. These give to the upper interface the properties of a two-dimensional gas. When the monolayer is compressed, some of the hydrophobic groups are driven from the upper interface into the interstratum. The irreversible formation of the monolayers indicates that the globular proteins have an entirely different structure and give support to the cyclol theory.

The viscosities and elasticities of monolayers furnish information regarding the cross linkages between the chains.

Stearic acid spread on water containing traces of barium salts gives monolayers which can be deposited by a dipping process upon solid plates. By successive dips any number of layers, up to 3000, can be built up. Optical measurements, involving interference of light reflected from the top and bottom surfaces, give accurately the thickness of the film.

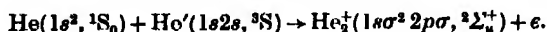
Single monolayers of various substances, deposited upon barium stearate multilayers of critical thickness (about  $1/4$  wave-length), are readily visible to the naked eye because of the change of colour. With monochromatic light the thickness of the monolayer can be measured to within about 2 Å.

The barium stearate multilayers are both hydrophobic and oleophobic (non-wettable by oil). Dipping into dilute solutions of thorium nitrate causes an overturning of the outside layer of molecules, making the surface polar and hydrophilic. Such conditioned surfaces can adsorb many organic substances from solution, giving observable increases of thickness. This technique serves as a valuable tool in biological investigations.

Free stearic acid in barium stearate multilayers can be dissolved out by dipping the film into benzene containing 1 % alcohol leaving a skeleton of unchanged thickness, but of refractive index which may be as low as 1.2. The application of a drop of oil fills the pores of the skeleton without wetting the surface and restores the original colour. Films of many substances may be deposited upon skeleton films and the permeability of the deposited film to liquids or vapours can thus be measured optically.

**The formation of helium molecules. II.** By F. L. ARNOT and M. B. M'EWEN.  
(Communicated by H. S. Allen, F.R.S.—Received 9 January 1939.)

An investigation of the formation of ionized helium molecules has been made by means of a mass spectrograph. The results obtained confirm the conclusion reached in our first paper on this subject from less definite evidence. It is shown that helium molecular ions are formed by the attachment of atoms in the  $1s2s$ ,  $^3S$  metastable state of 19.77 eV energy to normal atoms in the ground state  $1s^2$ ,  $^1S_0$  according to the process



Since the ground state of the neutral molecule is unstable this process fixes an upper limit of 19.77 V for the ionization potential of the molecule. The lower limits for the work of dissociation of the neutral molecule in its lowest stable state  $1s\sigma^2 2p\sigma 2s\sigma, ^3\Sigma_u^+$  and of the ionized molecule are found to be, respectively, 4.23 and 4.70 eV, which are both 1.7 eV higher than the usually accepted values.

Attention is drawn to the fact that these molecular ions are formed from excited atoms in S-states, whereas we have shown that mercury molecular ions are apparently formed only by excited atoms in P-states.

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**Utilization and digestion of carbohydrates by the adult blow-fly.** By G. FRAENKEL. (*Communicated by D. M. S. Watson, F.R.S.—Received 16 January 1939.*)

A series of different sugars and sugar alcohols has been fed to adult flies and their nutritional value determined by their effect on the longevity.

All the di- and tri-saccharides and glycosides which are of great nutritional value for the flies are split in the gut of the flies by enzymes, the presence of which could be demonstrated *in vitro*. No enzymes could be found by the same method which would split any of the non-utilized substances.

Weidenhagen's theory on the specificity of carbohydrases offers a full explanation of the results of the feeding and digestion experiments. The presence of only two enzymes,  $\alpha$ -glucosidase and  $\alpha$ -galactosidase, in the gut of the fly would account for the different action of different di- and tri-saccharides and glycosides.

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**Determination of the adiabatic piezo-optic coefficient of liquids.** By Sir VENKATA RAMAN, F.R.S., and K. S. VENKATARAMAN. (*Received 16 January 1939.*)

The piezo-optic coefficient defining the change of refractive index of liquids with pressure has till now been measured only for the case of isothermal compression. In such important applications, however, as the theory of the diffraction of light in ultrasonic fields and of the scattering of light in liquids by the Debye waves, it is the effect of adiabatically applied pressure on the refractive index which requires to be known. In the present paper an experimental technique is described which enables the adiabatic piezo-optic coefficients of liquids to be measured with standard laboratory equipment and actually with greater ease and precision than the corresponding isothermal coefficient. The results obtained for six common liquids are discussed with reference to (1) the deviations from the Lorentz refraction formula, (2) the variation of refractive index with change of temperature when the density is maintained constant, (3) the relation between the adiabatic and isothermal piezo-optic coefficients. These three matters are shown to be closely connected. The results and the discussion based thereupon show clearly that the present technique opens up a field of research which is of considerable interest in relation to the optical properties of liquids and their molecular structure.

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**The mechanism of polymerization reactions. I. The polymerization of styrene and methyl methacrylate.** By R. G. W. NORRISH, F.R.S., and E. F. BROOKMAN. (*Received 16 January 1939.*)

A study of the kinetics of the polymerization of methyl methacrylate and the co-polymerization of methyl methacrylate and styrene as typical vinyl compounds

has been made at various temperatures between 80 and 130° C. In the absence of a catalyst the reaction is slow and irreproducible, but with benzoyl peroxide or the ozonide of methyl methacrylate as catalyst the reaction is fast and of zero order. It is concluded that in the absence of added catalyst the reaction is dependent on the presence of adventitious catalysts. After between 10 and 20 % of the methyl methacrylate has polymerized the reaction becomes self-heating with a corresponding increase of velocity. This is correlated with the suppression of convection currents as the medium loses its fluidity and the consequent loss of isothermal conditions due to the poor conductivity and high heat of reaction. The velocity of co-polymerization is always between the velocities of polymerization of the two pure substances and follows a simple law.

Measurements of molecular weight have been made throughout the course of polymerization of methyl methacrylate and styrene in the presence of catalysts. With the former the molecular weight of the polymer grows steadily with the percentage polymerization. With the latter it first grows and then remains constant. In the case of styrene without catalyst, however, the molecular weight remains constant throughout the whole of the reaction.

These facts are correlated by a theory of the kinetics of polymerization in which the rate of reaction is limited by the rate of chain propagation rather than by the rate of initiation of chains. The theory is supported by the fact that the energy of activation in the case of methyl methacrylate is apparently independent of the nature of the two catalysts used. It explains adequately the varied observations on the molecular weight during polymerization. A mechanism of polymerization based on an earlier theory of Staudinger is proposed. The polymerization is supposed to be started by free radicals or other reactive centres derived from the catalyst and to be propagated by way of a free valence at the end of the growing chain. From time to time the growing chain may be terminated by hydrogenation at the expense of the monomer, but this results in the starting of a new chain by the monomeric free radical produced. In this way the number of reaction centres is maintained constant and proportional to the concentration of catalyst and the reaction is consequently of zero order.

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### **Synthetic oestrogenic compounds related to stilbene and diphenylethane.**

**Part I.** By E. C. DODDS, L. GOLBERG, W. LAWSON and Sir ROBERT ROBINSON, F.R.S. (*Received 17 January 1939.*)

Following the discovery of the oestrogenic activity of certain simple di-phenols such as 4 : 4'-dihydroxydiphenyl and 4 : 4'-dihydroxystilbene, a survey of analogous substances was instituted in order to study the relation between constitution and biological activity. Direction was given to this investigation by the selection of compounds the structure of which bears a close relation to that of oestradiol, and from this point of view attention was early concentrated on 4 : 4'-dihydroxy- $\alpha\beta$ -diethylstilbene and the dihydro-derivative of this substance.

A method of synthesis has been devised that enables the alkyl groups in the  $\alpha\beta$  positions to be varied, and a large number of dihydroxydialkylstilbenes has been prepared and examined for their oestrogenic activity.

Deoxyanisoin is alkylated, then submitted to the Grignard reaction and the product dehydrated. Demethylation is effected by means of alcoholic potassium hydroxide in an autoclave. The maximum activity is found in the diethyl-compound which is comparable with the natural hormones and even surpasses them.

Pinacols derived from *p*-hydroxyacylophenones are oestrogenic, and here again the pinacol from *p*-hydroxypropiofenone possesses the maximum activity for the series. This pinacol on dehydration affords a *diene* which is a remarkably potent oestrogenic agent and more so than its lower and higher homologues. It is particularly effective when administered orally.

The oestrogenic activity of certain naphthalene derivatives and of *trans*-dihydroxy-hexahydrochrysene has also been examined but these substances are not comparable with 4 : 4'-dihydroxy- $\alpha\beta$ -diethylstilbene or its dehydro-derivative, the above-mentioned *diene*.

A stereoisomeride of dihydroxydiethylstilbene has been isolated and characterized, and it is a less powerful oestrogenic agent than the isomeride.

On catalytic reduction the *diene* or this pseudo-derivative afford a dihydroxy-diethyldiphenylmethane, m.p. 184°C, which has been found in an independent research to be one of the most powerful oestrogenic agents known.

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**The protobranchiate mollusca; a functional interpretation of their structure and evolution.** By C. M. YONGE. (*Communicated by W. T. Calman, F.R.S.*—Received 19 January 1939.)

The Protobranchia represent the one unquestionably natural group within the class Lamellibranchia. The large foot permits movement into and through a soft substratum. Feeding, except in the modified Solenomyidae, is by means of the palp proboscis which collects organic detritus from the substratum. In these respects the structure of the Protobranchia probably corresponds closely to that of the primitive Lamellibranchia, the evolution of the palp proboscides permitting retreat of the mouth from the substratum and the enclosure of the body within the bivalve shell. This involved life on a soft substratum, so that the apparently specialized foot is probably primitive for the Lamellibranchia.

The Nuculidae are the most primitive of existing Protobranchia, having an anterior inhalent current, primitive ctenidia essentially respiratory in function, and hypobranchial glands. Conditions are essentially similar in the Solenomyidae, but the ctenidia are enlarged as feeding organs. The Nuculanidae possess highly specialized "pumping" ctenidia forming delicate septal membranes, the filaments being united by complex ciliated junctions to each other and to the siphonal septum. The inhalent current is posterior, and siphons occur which in different species show interesting stages in the conversion of ciliary into tissue junctions. Rejection of sediment from the mantle cavity is by cilia except in the Solenomyidae where it is brought about by the intucking of the ventral, uncalcified regions of the shell.

The alimentary canal is correlated in structure with the nature of the food particles collected by the palps. There is a dorsal crushing region lined with chitin and a ventral

style-sac" region which secretes mucus. Particles are embedded in this to assist trituration and the formation of faeces. There is no amylase and so no extracellular digestion. The digestive diverticula are organs of intracellular digestion. They consist of paired masses except in the Nuculanidae, where there is an additional mass of wide diverticula opening near the entrance of the oesophagus and into which larger particles enter. There are no wandering phagocytic cells. The intestine and rectum are concerned exclusively with the consolidation of faeces.

The nature, interrelationships, systematic position and evolution of the Proto-branchia are discussed.

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**The infra-red absorption spectrum of tetradeuteroethylene.** By G. K. T. CONN and G. B. B. M. SUTHERLAND. (*Communicated by R. G. W. Norrish, F.R.S.—Received 23 January 1939.*)

The infra-red absorption spectrum of tetradeuteroethylene has been observed between 550 and 3100  $\text{cm}^{-1}$  under high dispersion, the object being to discriminate (a) between different assignments of the fundamental frequencies of ethylene, (b) between different force fields which have been proposed to correlate the internal vibrations of the molecule. Strong absorption was found near 720, 1080, 2200 and 2350  $\text{cm}^{-1}$ , while weaker absorption took place near 1290, 1500 and 2970  $\text{cm}^{-1}$ . All of these bands exhibit sharp *Q* branches except that at 2350  $\text{cm}^{-1}$ , the contour of which it was impossible to obtain because of strong atmospheric absorption due to carbon dioxide. The band at 720  $\text{cm}^{-1}$  is particularly interesting in that the *Q* branch is double. The question of the assignment of the fundamental frequencies of ethylene is discussed in detail, and it is shown that none of the present assignments can be regarded as satisfactory. The correlation of the observed frequencies of tetradeuteroethylene with those of ethylene is achieved by use of the general equations for the isotope effect, and by comparison of the contours of the bands. In this way a new assignment of the frequencies of ethylene is arrived at which satisfactorily accounts for all the infra-red and Raman frequencies of both molecules. Since this new assignment differs from all previous ones it is possible to make the comparison between predicted and observed values of the isotopic frequencies only for those of the fundamental frequencies which have not been reassigned. A new descriptive notation for the twelve frequencies is introduced to avoid the confusion existing between present numerical notations.

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**Two-phase equilibria in binary and ternary systems.** By M. RUHEMANN. (*Communicated by A. C. Egerton, Sec.R.S.—Received 23 January 1939.*)

It is shown that the relations between temperature, pressure and composition for a binary liquid in equilibrium with its vapour in general cannot be calculated with the help of thermodynamical formulae containing only the thermal data of the pure components.

After a summary of existing methods for determining liquid-vapour equilibrium, an apparatus is described through which a mixture is passed slowly at a given temperature and pressure, whereby it is separated into two phases. The compositions of both phases are determined continuously with a catharometer.

Pressure-composition curves of methane-ethane mixtures were obtained at  $-104$ ,  $-88$ ,  $-78$ ,  $-42$  and  $0^{\circ}$  C at pressures up to 100 atm., and the results were used to calculate temperature-composition curves for 5, 10, 15 and 20 atm. The critical pressures of methane-ethane mixtures were found to be very much higher than those of either component, so much so that, in the pressure interval covered, a platt point could be observed only at  $0^{\circ}$  C. At  $-78^{\circ}$ , only  $4^{\circ}$  above the critical point of ethane, the critical pressure has risen from 45.8 atm. for pure methane to a value well above 100 atm. At low pressures methane-ethane mixtures show only slight deviations from perfect solutions.

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**The theory of crystal rectifiers.** By N. F. MOTT, F.R.S. (*Received 24 January 1939.*)

The existing theories of the action of crystal rectifiers assume that between the semi-conductor and the metal there is a potential barrier which the electrons penetrate by the tunnel effect. It is shown that this theory gives rectification in the opposite direction to that observed. An alternative theory is proposed; the nature of the potential barrier is discussed, and it is assumed that electrons have to be thermally excited so that they go over the barrier instead of through it. Good agreement with experiment is observed.

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**Cosmic rays at high altitudes.** By H. J. J. BRADDICK. (*Communicated by P. M. S. Blackett, F.R.S.—Received 25 January 1939.*)

Measurements of cosmic-ray bursts have been made in an aeroplane up to heights of 9.2 km. The number of large bursts is found to increase more rapidly than the number of small bursts, and both large and small bursts more rapidly than the number of showers recorded by counters. This is taken to indicate a change in the energy distribution of the electrons with height.

At a height of 9 km., the ratios of large bursts, small bursts, showers, and singles to the corresponding values at sea-level are approximately 125 : 1, 90 : 1, 57 : 1 and 18 : 1.

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**The propagation of a pulse in the atmosphere.** By C. L. PEKERIS. (*Communicated by G. I. Taylor, F.R.S.—Received 30 January 1939.*)

It is shown that in a composite atmosphere, such as was assumed in a previous paper in connexion with the theory of atmospheric tides, a surface pulse would excite waves of the first and second modes of oscillation, the amplitude of the former being greater than that of the latter by a factor varying from 2.4 to 2.9. This factor



would tend to increase on account of dispersion. Some records of the atmospheric wave which was caused by the Krakatoa eruption of 1883 are discussed with a view to identifying the wave of the second mode. There are indications of this wave in the first passage and, to a lesser degree, in the second passage. The energy of these waves is estimated to be of the order of  $10^{24}$  ergs. In the appendix is given the distribution with height of the vertical velocities in the two modes of oscillation of a model atmosphere. At heights of the order of 100 km., these velocities are found to be in phase with the surface pressure for both modes.

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**Coriolis perturbations in the methane spectrum. IV. Four general types of Coriolis perturbation.** By H. A. JAHN. (*Communicated by Sir William Bragg, O.M., P.R.S.—Received 1 February 1939.*)

In this paper a group-theoretical classification is made of the types of Coriolis perturbation which are possible in a tetrahedral molecule. It is shown that there are in all four distinct types of Coriolis perturbation of an infra-red active vibrational level. These arise from a Coriolis coupling of the rotational levels of this  $F_2$  type vibrational level with the rotational levels of  $A_1$ ,  $E$ ,  $F_1$  and  $F_2$  type vibrational levels respectively. The  $E-F_2$  type perturbation has been investigated in previous papers and here general formulae are derived for the matrix elements of the  $A_1-F_2$ ,  $F_1-F_2$  and  $F_2-F_2$  type perturbations. It is shown that the  $A_1$ ,  $F_1$  and  $F_2-F_2$  type perturbations do not produce a splitting of the individual rotational levels, the first causing a displacement of the  $Q$  branch levels alone and the second a displacement of the  $P$  and  $R$  branch levels alone. The  $F_1-F_2$  type like the  $E-F_2$  type perturbation produces a splitting as well as a displacement of the rotational levels. The energy matrix determining this splitting is factorized up to the tenth rotational quantum number with the help of group theory. The formulae given here form the necessary basis for an analysis of any infra-red absorption band of methane.

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**Cytochrome and cytochrome oxidase.** By D. KEILIN, F.R.S., and E. F. HARTREE. (*Received 2 February 1939.*)

The effects of respiratory inhibitors on cytochrome components as shown by changes in the appearances of their  $\alpha$ ,  $\beta$  and especially their  $\gamma$  bands reveal the existence of a new component,  $\alpha_2$ . The study of this component strongly supports the view identifying it with cytochrome oxidase.

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**The influence of nitrogen peroxide on the two-stage ignition of hydrocarbons.** By G. P. KANE. (*Communicated by A. C. Egerton, Sec.R S.—Received 2 February 1939.*)

In a study of the influence of  $\text{NO}_2$  additions on the spontaneous ignition of higher hydrocarbon-oxygen media, it has been found that its action depends upon the temperatures employed. At  $430^\circ\text{C}$ , i.e. above the temperature region where cool

flames are propagated,  $\text{NO}_2$  in concentrations up to 6 % always promotes the ignitions as is indicated by a reduction in the minimum pressures for spontaneous ignition and a shortening of the time lags. At lower temperatures, under circumstances involving a two-stage ignition, the effect of  $\text{NO}_2$  additions is of a composite nature consisting of a simultaneous inhibition of the reactions leading to cool flames and a promotion of the direct oxidation of the hydrocarbon.

With propane the inhibiting influence is marked by a lengthening of the cool flame and total time lags ( $t_1$  and  $(t_1 + t_2)$  respectively) with small  $\text{NO}_2$  additions, as well as an increase in the minimum pressure for the two-stage ignition. At a critical concentration of about 3.25 %  $\text{NO}_2$ , however, the cool flames are no longer observable; beyond this concentration, an overall promoting influence induced by the direct oxidation becomes evident and is characterized by abrupt ignitions at greatly reduced minimum pressures and shorter time lags.

With *n*-butane, the inhibition is indicated by a continuous increase in the time lags with  $\text{NO}_2$  additions and elimination of the cool flames when more than 5 % of it is added. The promotion of the direct oxidation by  $\text{NO}_2$  is greater with *n*-butane than with propane, and results in a lowering of the minimum pressure for the two-stage ignition with small  $\text{NO}_2$  additions; with large amounts of  $\text{NO}_2$  an anti-catalytic influence becomes evident probably as a result of self-neutralization of active centres.

A similar influence is observed with acetaldehyde. As the inhibition of the cool flame reaction is greatest with acetaldehyde the latter material is considered to be of primary importance in the formation of cool flames.

The behaviour of nitrogen peroxide is interpreted in relation to the promotion of a primary direct oxidation  $A \rightarrow C$ , which occurs mainly at high temperatures, and the inhibition of a two-stage reaction  $A \rightarrow B \rightarrow C$ , predominant at lower temperatures by a process of neutralization of the active intermediate product *B*. The observed specific influence of  $\text{NO}_2$  on these different stages in the combustion of higher hydrocarbons is discussed and the bearing of these results on "knock" is indicated.

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**The monaural threshold: the effect of subliminal and audible contralateral and ipsilateral stimuli.** By J. W. HUGHES. (*Communicated by T. Graham Brown, F.R.S. —Received 3 February 1939.*)

Application of contralateral subliminal stimuli decreases the monaural threshold, whatever the relative frequencies of the two notes used. The decrease is such that the sum of the energies applied to each ear, expressed as fractions of the corresponding monaural threshold energies, is unity. Ipsilateral subliminal stimuli also decrease the threshold, but less than do corresponding contralateral stimuli.

For two notes differing considerably in pitch the monaural threshold is unaffected by contralateral audible stimuli of intensities up to 15 db. above their threshold. No definite threshold variation occurred when similar audible ipsilateral stimuli were applied.

The explanations previously given for the first results are modified, and central summation is discussed.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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3 APRIL 1939

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**Absorption of electromagnetic waves in the earth's atmosphere.** By R. R. BAJPAI and K. B. MATHUR. (*Communicated by M. N. Saha, F.R.S.*—Received 17 January 1939.)

The paper gives an analytical treatment of the absorption of the two magneto-ionic components of radio waves in the upper atmosphere for all the magnetic latitudes. General expressions for refractive index and absorption coefficient in the quasi-longitudinal and quasi-transverse types of propagation have been derived and discussed. Curves showing the variation of the regions of the validity of the Q.L. and Q.T. approximations with magnetic latitudes have been drawn for five different frequencies. It is seen from these figures that in the non-reflecting region Q.T. approximations are valid for places very near the magnetic equator while Q.L. approximations hold in the rest of the places; it has been shown that at a particular level in the former region both the waves are almost equally absorbed but in the latter, the *o*-wave suffers less absorption than the *n*-wave. The curves further show that for propagation in the reflecting regions the entire surface of the earth can be divided up into three regions: (1) the magnetic equatorial latitudes, (2) the intermediate magnetic latitudes, and (3) the high magnetic latitudes. It has been shown that at a particular level in case (1)  $k_o < k_n$  according as  $p < p_h$ , but in case (2)  $k_o > k_n$  and in case (3)  $k_o < k_n$ , irrespective of the fact whether  $p < p_h$ .

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**The atomic heat of liquid helium below 1°K.** By G. L. PICKARD and F. SIMON. (*Communicated by F. A. Lindemann, F.R.S.*—Received 24 January 1939.)

Details are given of a recently constructed helium liquefier using the expansion method. In this apparatus the "capsule" technique, which is described in some

detail, has been used to cool liquid helium to low temperatures by the magnetic method.

The atomic heat of liquid helium has been measured between 0.25 and 1°. It is shown that the anomalous specific heat due to the  $\lambda$ -phenomenon dies out just below 1°, and at lower temperatures the specific heat may be represented by a Debye Function with a  $\theta$  of 17°. Further reasons are given supporting the view that below about 0.8° the specific heat is entirely due to thermal vibrations.

**On the thermodynamics of moving matter. I. Phenomenological theory.**

By D. VAN DANTZIG. (*Communicated by P. A. M. Dirac, F.R.S.—Received 6 February 1939.*)

The paper contains an outline of the phenomenological thermodynamics of moving matter, more in particular of perfectly perfect fluids, with special regard to the invariance under arbitrary transformations of co-ordinates in space-time, and to the independence of metrical geometry.

It is shown that the fundamental equations can be brought into an invariant form which holds in classical as well as relativistic physics. This can be done by means of the temperature vector, the time component of which is the reciprocal absolute temperature and which is directed along the macroscopical world-lines, and the vector of average momentum and enthalpy pro particle. The fundamental invariant, called "thermasy", is the time integral of the absolute temperature.

As a special result a relativistic effect (too small to be observable) is found, stating that a temperature gradient in a flowing imperfectly perfect fluid (e.g. liquid helium) gives rise to internal friction.

**The equations of motion of point electrons deduced from a variational principle.**

By T. LEWIS. (*Communicated by E. A. Milne, F.R.S.—Received 7 February 1939.*)

The equations of motion of point electrons are derived on the assumption that they move in such a way as to make the integral  $I = \iiint (H^2 - E^2) dx dy dz dt$  stationary as compared with its value for neighbouring motions which leave the lengths of small elements of the world lines of electrons unchanged. The (rest) mass of an electron enters as a Lagrangian multiplier and turns out to be constant. This method leads to equations which are identical in form with those obtained from Fokker's variation principle. The equations of motion of a single electron in an electrostatic field are shown to be those in general use for the relativity electron. The resisting force due to radiation, which is a consequence of the electromagnetic theory of mass, is absent in the present theory, which differs in this respect from those of Dirac and Pryce.

**Studies on the reproduction of the dogfish.** By H. METTEN. (*Communicated by H. Graham Cannon, F.R.S.—Received 9 February 1939.*)

For abstract, see *Nature, Lond.*, 143, 121, 21 January 1939.

**Some factors producing individual differences in dark adaptation.** By L. R. PHILLIPS. (*Communicated by C. Lovatt Evans, F.R.S.—Received 13 February 1939.*)

By means of the light threshold method, with circumfoveal observation and white intermittent stimuli of  $\frac{1}{2}$  sec. duration, the course of dark adaptation was traced over a period of 20 min. in two groups of forty and twenty-six observers.

By correlating measures of pupil diameter, general colouring, visual acuity in the light, retinal pigmentation and age with the corresponding perception times for selected test patch brightnesses, and by estimating the significance of the regressions over the whole of the data, the influence of these factors was determined.

The first group, males of approximately 20 years of age previously dosed to eliminate vitamin A deficiency, showed the influence of pupil diameter in the light, and in the dark, to be highly significant. A comparatively large pupil in the light retarded dark adaptation, whilst a large pupil diameter in the dark accelerated it. By the same method, the influence of general colouring and visual acuity in the light was shown to be negligible, but there was some evidence that high retinal pigmentation favoured more rapid dark adaptation.

In the second group, males and females, aged 17–70 years, increase in age was shown significantly to retard dark adaptation. This effect was not due to changes in the diameter of the pupil in the light with age, but in part to a decrease in the pupil diameter in the dark.

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**Deviation from the Coulomb law for the proton.** By H. FRÖHLICH, W. HEITLER and B. KAHN. (*Communicated by N. F. Mott, F.R.S.—Received 13 February 1939.*)

We have calculated the deviation from the Coulomb law of force for the proton on grounds of the mesotron theory of the nuclear particles. The effect is due to the fact that the proton spends a certain fraction of its life in a dissociated state as a neutron and a positive mesotron, the latter having an average distance from the neutron of the order of magnitude of the electronic radius  $r_0$ . The result is the following: The Coulomb attraction (for a negative point charge) goes over into a strong repulsion for distances less than about  $1/6r_0$ .

This would lead to a shift of the hydrogen *S*-levels towards higher energies of the order of  $1 \text{ cm.}^{-1}$  for the  $1S$ -level and one-eighth of this figure for the  $2S$ -level. This is compared with recent experiments by R. C. Williams on the fine structure of the  $D_\alpha$  and  $H_\alpha$  line which, according to Pasternack, can be explained by assuming a shift of the  $2S$ -level by  $0.03 \text{ cm.}^{-1}$ .

How far these results lie within the limits of validity of present quantum mechanics is discussed.

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**On the photo-ionization of molecules.** By B. D. NAG CHOWDHURI and K. BASU. (*Communicated by M. N. Saha, F.R.S.—Received 13 February 1939.*)

In this paper an attempt has been made to calculate theoretically a formula for  $\tau(\nu)$ , the absorption coefficient indicating photo-ionization of molecules. The particular case attempted is that of  $\text{H}_2$ , and for the transition  $1s^2 \rightarrow 1s\sigma\sigma$ . The  $\text{H}_2^+$ -ion plus the

free electron has been regarded as consisting of two H-atoms, one in the normal  $1s$ -state, the other in the state  $\cos$ , which indicates an electron describing a hyperbolic orbit with  $l$ , the angular momentum = 0. This case has been selected for its simplicity, though the actual absorption corresponds to  $1s^1 \rightarrow 1s\sigma p$ , i.e. the hyperbolic orbit of the free electron should have  $l = 1$ . No solution of this case has yet been obtained.

The case is further complicated by the fact that the Franck-Condon principle has to be taken into account, as the nuclear distances of the normal  $H_2$ -atom and  $H_2$ -ion are widely different.

The actual formula for  $\tau(\nu)$  has a very complicated form (vide formulae (34.1) and (44)) and the  $\tau(\nu)/\nu$ -curve is shown in Fig. (2). It consists of a series of discontinuous strips, starting from  $\nu = \nu_0$ , the least ionization-frequency, and jumping discontinuously at each point,  $\nu = \nu_0 + v'\omega'_0$  where  $\omega'_0$  is the fundamental vibration frequency of the  $H_2^+$ -ion. The maximum value of  $\tau(\nu)$  is reached at  $\nu = \nu_0 + 2\omega'_0$  for the particular transition considered in this paper.

It is considered probable that the general form of the photo-ionization curve for diatomic molecules will be of this form.

**The development of the vertebral column and Weberian ossicles of the goldfish (*Carassius auratus*).** By J. M. WATSON. (*Communicated by E. W. MacBride, F.R.S.* - Received 14 February 1939.)

The early stages of the development and formation of the vertebral column and Weberian ossicles in the goldfish (*Carassius auratus*) are described from the time of hatching till the adult condition is acquired at 25 mm. and it is concluded that: the centrum arises by ossification first of the fibrous sheath and later of the perichordal sheath; cartilaginous basidorsals and mesenchymatous basiventrals and haemapophyses are present in the posterior unmodified vertebrae; neither dorsal nor ventral intercalaries occur, the vertebral column being essentially monospondylous, even in the anterior region where there is some modification owing to the presence of the Weberian ossicles; supradorsals are lacking; the anterior zygapophyses arise as processes of the neural arch, and the posterior zygapophyses as processes of the perichordal sheath; the spinal cord in the region of the first vertebra (which lacks a neural arch) is roofed over by a backward extension of the exoccipitals; the basidorsals of the second (part only), third and fourth vertebrae fuse with the first three interspinous bones to form a massive arch of cartilage which later gives rise to the neural arches and neural spines of the compound vertebra and the fourth vertebra; the transverse processes of the first and second vertebrae are homologous with the dorsal ribs of Selachii; the centra of the second and third vertebrae fuse to form a compound centrum; the ossa suspensoria are modified haemapophyses; the claustrum arises as a direct ossification of the connective tissue forming the wall of the atrium sinus imparis; the scaphium arises in part from the basidorsal of the first vertebra, in part from an independent mesenchymatous rudiment; the intercalarium arises in part from the basidorsal of the second vertebra, in part from an ossification of the interossicular ligament; the tripus arises in part from the basiventral of the third

vertebra and in part from an independent mesenchymatous rudiment, together with a small ossification of the interossicular ligament and a small ossification of the outer coat of the air-bladder; the condition of the embryonic Weberian chain in the goldfish is so similar to the adult condition in the Siluroidea that in all probability the indirect cyprinoid system has evolved from the direct siluroid system.

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**The effect of electrolytes upon the interfacial tension between water and dekaline (*trans*-decahydronaphthalene).** By W. L. GUEST and W. C. M. LEWIS, F.R.S. (*Received 16 February 1939.*)

Employing the drop-volume stalagmometer as developed by Dedrick and Hanson the interfacial tension between dekaline (*trans*-decahydronaphthalene) and aqueous solutions of the following salts has been determined as a function of concentration at 25° C: KCl, LiCl, BaCl<sub>2</sub>, LaCl<sub>3</sub>, AlCl<sub>3</sub>, KI, KSCN, LiI, and LiSCN. The last four salts are capillary active in the sense that the interfacial tension falls with increase in concentration of the active individuals, namely the iodide and thiocyanate ions. Such capillary activity is specific, there being no theoretical explanation available at the present time to account for the behaviour.

Of the five chlorides examined, two of them, namely, LiCl and LaCl<sub>3</sub>, exhibit a minimum in interfacial tension in the dilute region followed by a rise beyond that given by water alone in contact with dekaline. The existence of the minimum is accounted for, in a formal manner at any rate, by the theory advanced by Dole to account for the minimum observed by Jones and Ray at a region of still greater dilution (0.001 N) in the case of simple salts at the air-water surface. In the case of KCl and BaCl<sub>2</sub> which apparently do not exhibit a minimum, the influence exerted upon the interfacial tension is so minute (at great dilution) that the existing experimental technique is not sufficiently precise to permit of the application of the theory of Dole. In the region of higher concentration where the chlorides referred to exhibit an increase in tension with increase in concentration the discrepancy between the values observed and those calculated (either on the theory of Dole or on that of Wagner-Onsager-Samaras) is similar to that exhibited by simple salts at the air-water surface.

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**Further studies on pre-imaginal olfactory conditioning in insects.** By W. H. THORPE. (*Communicated by A. D. Imms, F.R.S.—Received 20 February 1939.*)

Previous work on positive pre-imaginal olfactory conditioning of the ichneumonid parasite *Nemeritis canescens*, by exposure to the odour of unusual chemical substances, has been repeated using a pure strain of *Drosophila melanogaster*. This latter insect has certain advantages as an experimental animal, the first of which is the absence of a cocoon. Owing to this it is impossible to wash the surface of the larvae free from all food and other contaminating substances prior to pupation and thus obtain more reliable evidence as to conditioning resulting from treatment of the larval stage only. The second advantage is that the rearing of *Drosophila* on an artificial

culture medium is a standardized process and the substance to be tested can be conveniently mixed with the medium. 0.5 % of peppermint essence (containing 4.6 % free menthol) was found to be satisfactory.

All the main conclusions established by the work on *Nemeritis* have been confirmed even more strikingly with *Drosophila*. Although normally repelled by the odour of peppermint, flies which have been reared on a medium containing 0.5 % peppermint essence are markedly attracted by the odour of this substance in an olfactometer. Conditioning brought about in this way gradually becomes extinct if the insects are isolated and has practically disappeared in about 6 days. Washing the fully-fed larvae, or newly-formed puparia, free from the medium does not eliminate the conditioning effect although it reduces it to some extent. It is, therefore, concluded that a change in the responses of the adult can be brought about by an influence operating only during the larval life.

It has been shown that with *Drosophila*, as with *Nemeritis*, exposure of the adult to the odour of the substance immediately after emergence from the pupa will bring about a positive conditioning even though the odour is not specifically associated with any particular favourable quality of the environment.

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**High-altitude cosmic radiation measurements near the north geomagnetic pole.** By H. CARMICHAEL and E. G. DYMOND. (*Communicated by E. V. Appleton, F.R.S.—Received 22 February 1939.*)

Measurements of cosmic radiation have been made at very high altitudes near the north geomagnetic pole with two kinds of apparatus, namely, a triple coincidence apparatus with radio transmission of the data and an ionization apparatus which was self-recording. The lowest atmospheric pressure reached by the vertical coincidence apparatus was 52 mm. Hg, and by the ionization apparatus 12.5 mm. Hg. Comparison of our results with those obtained by others between geomagnetic latitudes 49 and 60° N. shows that no appreciable quantity of low-energy cosmic radiation is reaching the earth from outer space. In particular, the very good agreement between the shape of the curve showing ionization against pressure at geomagnetic latitude 85° N. and similar curves of Millikan and his co-workers at geomagnetic latitudes 55 and 60° N. indicates a complete absence of incoming radiation in the energy range from  $3 \times 10^7$  eV to about  $10^9$  or  $2 \times 10^9$  eV. Possible causes of the absence of such low-energy rays are discussed.

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**The thermal decomposition of diethyl ethyl.** By J. G. DAVOUD and C. N. HINSHELWOOD, F.R.S. (*Received 22 February 1939.*)

The thermal decomposition of diethyl ether yields acetaldehyde and a mixture of hydrocarbons, the acetaldehyde subsequently splitting into carbon monoxide and methane. In so far as the reaction of the aldehyde can be regarded as much faster than that of the ether the total decomposition can be represented approximately as a one-stage unimolecular reaction of the ether. On this basis the kinetics of the reaction have been investigated in the past. The exact degree of validity of the approximation has been uncertain.



In the present work analyses have been made of the amount of aldehyde present at each stage of the reaction, together with complete analyses of the gaseous products. These measurements allow the older determinations of the reaction rate to be subjected to correction so as to represent more accurately the true initial decomposition of the ether. In this way fresh investigations have been made both of the normal reaction involving radical chains, and of the non-chain reaction occurring in the presence of a few millimetres of nitric oxide. The part played by a small fraction of surface reaction has also been studied.

The dependence upon the initial ether pressure of the reaction rate has been redetermined, and the relation between nitric oxide inhibition and ether pressure has been further investigated.

The general conclusion is reached that previously determined velocity constants should be somewhat increased in absolute value, but that general deductions about the kinetics of the reaction are confirmed and need no essential correction.

An estimate has been made of the concentration of free radicals prevailing during the reaction in the absence of nitric oxide.

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**K-electron capture, nuclear isomerism and the long-period activities of titanium and scandium.** By H. WATKINS, E. J. WILLIAMS and G. R. EVANS. (*Communicated by J. Chadwick, F.R.S.—Received 22 January 1939.*)

A study has been made of the long-period activities induced in titanium and scandium by activation with deuterons of high energy and in titanium by activation with  $11 \times 10^6$  eV  $\alpha$ -particles.

A radioactive isotope of vanadium has been discovered of half-life  $600 \pm 50$  days which decays almost entirely by K-electron capture. It is ascribed to  $V^{47}$ . The ratio of K-capture to positron emission has been studied in  $V^{48}$ . The observed X-ray intensity is compatible with that required by the Fermi interaction, the ratio K-capture/positron emission being  $0.40 \pm 0.15$ .

An isomeric form of  $Ti^{51}$  has been detected of half-life  $72 \pm 2$  days which decays by emitting electrons of maximum energy  $0.36 \times 10^6$  eV, the product nucleus  $V^{51}$  being always left in an excited state of energy  $1.0 \times 10^6$  eV.

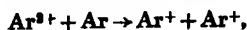
The branching decay of  $Sc^{46}$  by K-electron capture and electron emission has been observed. The half-life of  $Sc^{46}$  has been confirmed as  $85 \pm 1$  days, the maximum energy of the disintegration electrons being  $0.26 \times 10^6$  eV.  $\gamma$ -radiation of energy  $0.93 \times 10^6$  eV is also emitted.

A positron activity of half-life  $450 \pm 50$  days produced in titanium by  $\alpha$ -particle bombardment is provisionally assigned to  $Cr^{51}$ . The upper limit of the positron spectrum is at  $0.34 \times 10^6$  eV.

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**Electron transfer in argon.** By F. L. ARNOT and W. D. HART (*Communicated by H. S. Allen, F.R.S.—Received 24 February 1939.*)

An investigation of the electron transfer process



has been made by a new method using incident  $Ar^{3+}$  ions having energies from 162 to 404 eV. For each of these energies the forward-moving  $Ar^{+}$  ion has an energy,

due to its velocity component in the direction of the incident  $\text{Ar}^{2+}$  ion, of 13 eV less than that of the  $\text{Ar}^{3+}$  ion.

It is shown that the angle between the direction of the forward-moving  $\text{Ar}^+$  ion and that of the incident  $\text{Ar}^{2+}$  ion is about  $15.2$ ,  $10.6$  and  $7.4^\circ$  respectively for energies of the  $\text{Ar}^{2+}$  ion of 100, 200 and 400 eV. These angles of scattering are considerably greater than those for the corresponding process in mercury which are less than  $5$ ,  $3.6$  and  $2.5^\circ$  respectively for  $\text{Hg}^{2+}$  ions of energy 100, 200 and 400 eV.

The cross-section for the process over the energy range of 162–404 eV has been obtained, and this shows a linear increase of cross-section with energy of the incident  $\text{Ar}^{2+}$  ion, the value for 404 eV  $\text{Ar}^{2+}$  ions being about one-hundredth of the gas-kinetic cross-section for collision. Extrapolation of the cross-section curve gives the activation energy as 76 eV.

Attention is drawn to a numerical error made in determining the cross-section of the corresponding electron transfer process in mercury, previously reported. The ordinates of this curve should all be multiplied by a factor of 2. With this correction, the value of the cross-section for the mercury process is greater than one-tenth of the gas-kinetic cross-section over the entire energy range used, 140–400 eV.

**The asymptotic expansion of integral functions defined by Taylor series.**  
By E. M. WRIGHT. (*Communicated by G. H. Hardy, F.R.S.—Received 27 February 1939.*)

The function considered is  $f(x) = c_0 + c_1x + c_2x^2 \dots$ ,

where 
$$c_n = \frac{\phi(n)}{\Gamma(\kappa n + \beta)},$$

$\kappa$  and  $\beta$  may be real or complex,  $K(\kappa) > 0$  and  $\phi(t)$  is regular and has an asymptotic expansion in descending powers of  $t$  to the right of the line  $K(\kappa t) = h_1$ .

If  $K(1/\kappa) < \frac{1}{2}$ , the function has one or more exponentially large expansions for all large  $x$  in the complex  $x$ -plane. If  $K(1/\kappa) > \frac{1}{2}$ , the plane is divided by two spirals into two connected parts; in the interior of one part an exponentially large expansion is valid, in the other the expansion is algebraic, while in the neighbourhood of the spirals the expansion is mixed, i.e. a sum of the expansions in the two regions. If  $K(1/\kappa) = \frac{1}{2}$  the spirals coincide; there is an exponentially large expansion at a distance from the spiral and a mixed expansion in the neighbourhood of the spiral.

The results for the single expansion are similar to Watson's, but our conditions on  $\phi(t)$  are much less severe than his. The results for the mixed expansions are entirely new. Amongst other uses, the latter would enable us to determine the distribution of the zeros of the function very precisely.

Particular examples of the function have been studied by Mittag-Leffler, Barnes, Hardy and others. The results here include theirs as special cases.

The calculation of the coefficients in the exponential expansion is greatly shortened and the results are given in a simple form which should facilitate applications to particular problems. The method of proof is based on Cauchy's Theorem; while it has certain points in common with the method of steepest descent, the complications of the latter method are wholly avoided.

# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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**Upper air investigations in north-west Greenland.** By H. CARMICHAEL and E. G. DYMOND. (*Communicated by C. G. Barkla, F.R.S.—Received 24 February 1939.*)

The winds on the coast of north-west Greenland have been investigated during the summer, 1937, and a meteorograph sounding to a height of 19·5 km. has been made in latitude 76° N. Large balloons were used as pilots, and their rate of ascent was determined directly by double theodolite observations. The average bursting height of the balloons was 24·5 km.

The meteorograph revealed a well-marked tropopause at 11·2 km. and an unusual rise of temperature in the stratosphere. The pilot balloons showed very still air in the stratosphere throughout the summer and a marked velocity maximum at 9 km. in the troposphere.

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**On lunar tides in the upper atmosphere.** By E. V. APPLETON, F.R.S., and K. WEEKES. (*Received 1 March 1939.*)

Using the well-known radio methods of upper-atmospheric exploration, a lunar tide has been detected in the Kennelly-Heaviside layer (region E) of the ionosphere. The magnitude and phase of the tide may be specified by the statement that the experimentally determined expression for the equivalent height of the lower boundary of the layer is found to contain a term of the form

$$0\cdot93 \sin (2t' + 112^\circ) \text{ km.,}$$

where  $t'$  is the lunar hour angle. The tide is thus semidiurnal, of the order of 1 km. and attains its maxima about  $\frac{1}{2}$  hr. before the lunar transits. To the accuracy of the

experimental results, it is thus in phase with the lunar barometric pressure oscillations, as determined by Chapman for ground-level at Greenwich.

Difficulties are encountered in reconciling the new results with what has previously been deduced from other geophysical evidence concerning the magnitude and phase of upper-atmospheric oscillations.

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**Natural convection in liquids.** By O. A. SAUNDERS. (*Communicated by Sir Henry Tizard, F.R.S.—Received 1 March 1939.*)

In the first part of the paper theoretical solutions are given for the heat transfer by natural convection from a heated vertical plate to a fluid when the motion is streamline. The results agree with the now well-established experimental data for air, and also with the only experiments for a vertical plate in a liquid, namely those by Lorenz for oil.

The theory shows that the heat transfer,  $H$ , as expressed by the Nusselt number,  $Hl/k\theta$ , depends mainly on the product of the Grashof number,  $Gr (\equiv Bg\theta l^3/\nu^2)$  and the Prandtl number,  $Pr (c\nu/k)$ . For a fixed value of this product, the Nusselt number varies only 10 % over the range of Prandtl numbers for all gases and liquids, namely, approximately 0.66–0.9 for gases and 2.0 to several hundreds for liquids. The only exception is mercury, for which  $Pr$  is 0.03 and the theoretical Nusselt number is about 40 % below the value for air.

The second part of the paper describes experiments to measure the heat loss from vertical plates in mercury and in water. For streamline motion the results, both for mercury and for air, agree well with the theory.

The experiments with water were continued into the turbulent region, an optical method being used to show the character of the motion. It was found that the streamline motion first breaks down at the top of the plate for the same value of  $Gr \times Pr$  as found previously for air, namely, about  $2 \times 10^9$ . Formulae are given for the heat transfer when the flow is turbulent, showing how the Nusselt number varies with  $Gr$  and  $Pr$  for water and for air.

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**X-ray crystal analysis of trans-azobenzene.** By J. J. DE LANGE, J. MONTEATH ROBERTSON and I. WOODWARD. (*Communicated by Sir William Bragg, P.R.S.—Received 3 March 1939.*)

The crystal structure and molecular dimensions of the ordinary or *trans*-form of azobenzene have been determined by quantitative X-ray investigation. The crystal is isomorphous with the dibenzyl series of structures, and consequently it is possible to make a direct application of the Fourier series method, without assuming any new model for the azobenzene molecule. The resolution of the nitrogen atoms is poor, but the  $N=N$  distance of  $1.23 \pm 0.05$  Å is confirmed. The  $C-N$  distance is  $1.41 \pm 0.03$  Å, implying conjugation between the benzene ring and the  $N=N$  bond. The  $N=N-C$  valency angle is  $121.5 \pm 3^\circ$ . These results are discussed in relation to other investigations.

Two independent molecules contribute to the asymmetric crystal unit. One is practically flat, but in the other the rings lie in different planes 0.32 Å apart. This departure of the second molecule from the planar form is accompanied by small changes in dimensions. The orientations of the molecules and co-ordinates of the atoms relative to the crystal axes are given. The minimum intermolecular approach distances lie between 3.5 and 3.6 Å.

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**Effusion and thermal transpiration in quantum statistics.** By B. N. SRIVASTAVA. (*Communicated by Professor M. N. Saha, F.R.S.—Received 7 March 1939.*)

This paper is a continuation of the author's previous work in which the molecular effusion of matter into vacuum was considered. In the present work the molecular effusion of gas into another chamber containing gas at a different pressure or at different temperature is investigated and it is shown that the effusion from chamber 1 to 2 is equal to the effusion of gas 1 into vacuum minus the effusion of gas 2 into vacuum. In § 1 the effusion of one gas into vacuum at the same temperature but at different pressure has been calculated for the different cases. Thermal transpiration has been discussed in subsequent sections, and expressions have been worked out for the different cases giving the relation between the equilibrium pressures that will obtain in the steady state between two chambers maintained at different temperatures and communicating with each other through a narrow opening. For the non-degenerate and completely non-relativistic case it is found that the equilibrium pressure ratio becomes exaggerated for matter obeying Bose-Einstein statistics and decreased for matter obeying Fermi-Dirac statistics as compared to the value for a classical perfect gas. For the completely relativistic and completely non-degenerate case the pressures are found to be inversely as the temperatures. For degenerate matter in both chambers, degenerate in the sense of Fermi-Dirac statistics, the pressures are found to be equal to a first approximation but on working to a higher approximation, the pressure in the hotter vessel is found to be slightly greater. The relation between the pressures when matter in one chamber is degenerate and in the other non-degenerate has also been calculated.

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**Thermodynamics of the thermomechanical effect of liquid He II.** By H. LONDON. (*Communicated by N. F. Mott, F.R.S.—Received 7 March 1939.*)

The effect discovered by Allen and Jones that two reservoirs filled with He II and in communication through a capillary show a difference in hydrostatic pressure, if they are kept at different temperature, is treated thermodynamically. Considering a cycle in which the pressure difference is used to do mechanical work, one finds that reversible thermal effects must appear which are analogous to the Peltier effect.

Cold is produced where liquid He II leaves a capillary and heat where it enters. Both are given by the equation

$$Q = vT(\partial p/\partial T)_{p,A,T_A},$$

where  $v$  is the volume of liquid flowing through the capillary,  $T$  the absolute temperature and  $(\partial p/\partial T)_{p,T}$ , the variation of pressure with temperature in one vessel, if the other is kept at constant pressure and temperature.

The hypothesis of Tisza is discussed, according to which the atoms moving through a capillary are apart from lattice vibrations in the lowest energy state. The assumption is made that this process takes place reversibly. One obtains the equation

$$\rho s = (\partial p/\partial T)_{p,A,T_A},$$

where  $\rho s$  is the excess of the total entropy per cm.<sup>3</sup> of liquid helium above the part of the entropy due to lattice vibrations. This relation can be used for the experimental verification of the hypothesis.

Considering the circulation of liquid set up by the thermomechanical pressure a possible mechanism of heat transport is discussed which allows the correlation of the heat conduction experiments with those on the flow of liquid helium.

**The electric strength of gases measured by corona discharge.** By W. M. THORNTON. (*Communicated by Sir Oliver Lodge, F.R.S.—Received 7 March 1939.*)

When the electrical potential of a wire surrounded by a gas at or near atmospheric pressure is slowly raised, a glow or corona appears on the wire as if the surface became suddenly luminous. This is the first stage of spark discharge in the gas, which is prevented from developing farther by the rapid divergence of the field. The electric gradient at which it occurs is much higher than that at which a spark passes in a uniform field of force, but the latter can be derived from measurements of the corona, starting voltage, the radius of the wire and the density of the gas. In the present work the electric strength of some fifty gases has been found in this way. When set down against the reciprocal of the electron mean free path the gases are found to fall into several groups which lie on straight lines converging to the origin. In each of these the product of the electric strength and the free path is constant. This is interpreted to mean that the energy of ionization by collision that starts spark discharge is constant in each group of gases. Since the free path is inversely proportional to the cross-sectional area of the molecules it follows that the molecular areas of gases that have the same electric strengths in the several groups differ by finite and nearly equal steps. The electric strengths of gases having the same molecular area change only by such steps. Chlorine has the greatest strength of the elements examined, the mono-atomic gases the least. The paraffin series is most uniform; its chlorine substitution compounds show a regular change and reach remarkable values.

**The association of carcinogenicity and growth-inhibitory power in the polycyclic hydrocarbons and other substances.** By A. HADDOW and A. M. ROBINSON. (*Communicated by E. L. Kennaway.—Received 9 March 1939.*)

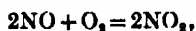
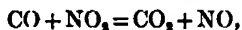
The results are given for 348 experiments in which 96 compounds were tested for their action on the growth of the Walker rat carcinoma 256, the Crocker mouse sarcoma 180, or spontaneous mammary cancer of the mouse. 86.5 per cent of 171 experiments with 34 carcinogenic substances resulted in growth inhibition, while 79.7 per cent of 79 experiments with 34 non-carcinogenic compounds gave no trace of inhibition. There is therefore a close statistical association of the biological properties of carcinogenicity and growth-inhibitory power.

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**A study of sensitized explosions. IV. The carbon monoxide-oxygen reaction catalysed by nitrogen peroxide.** By E. J. BUCKLER and R. G. W. NORRISH, F.R.S. (*Received 10 March 1939.*)

It has been found that nitrogen peroxide does not sensitize the ignition of pure dry carbon monoxide and oxygen, but that under the combined influence of nitrogen peroxide and hydrogen explosions occur over 300°C below the temperatures required for ignition of the pure gases. The sensitized explosions are preceded by induction periods which at first decrease and then increase with increasing concentrations of nitrogen peroxide. The dependence of induction periods on concentration of hydrogen and presence of inert gases has been studied and supports the view that explosion of  $\text{CO-O}_2\text{-NO}_2\text{-H}_2$  mixtures is due to a chain branching mechanism similar to that occurring in the  $\text{NO}_2$ -sensitized ignition of hydrogen and oxygen. At 500°C explosions also occur in  $\text{CO-O}_2\text{-NO}_2$  mixtures containing water vapour, by a chain mechanism in which it has been suggested that water catalyses chain propagation while nitrogen peroxide is responsible for chain branching.

The work of Crist and others has shown that nitrogen peroxide induces steady oxidation in mixtures of pure  $2\text{CO} + \text{O}_2$  at 750 mm. total pressure and 527°C, and that its catalytic action falls into two parts: (1) a catalysis governed by the two homogeneous reactions



which are important when the concentration of  $\text{NO}_2$  exceeds about 1.2 mm.; (2) a catalysis at lower concentrations of  $\text{NO}_2$  which passes through a maximum and has the characteristics of a chain reaction. These results have been confirmed using mixtures purified from traces of hydrogen. It was shown that the catalytic and anticatalytic effects of small concentrations of nitrogen peroxide in this reaction is not dependent on the presence of traces of hydrogen. However, in view of the extreme sensitivity of the reaction to the presence of the free radicals  $\text{H}$  and  $\text{OH}$  derived from water, which are known to catalyse the propagation of straight chains, it is believed that the catalysis has its origin in a branching reaction introduced by the nitrogen peroxide, identical in characteristics with that operating in the catalysis

of oxy-hydrogen mixtures by nitrogen peroxide, i.e. by its dissociation through participation as a third body in some exothermic link of the chain. Also, if complete dryness were possible it is to be expected that the catalytic and anticatalytic effects of  $\text{NO}_2$  at small concentrations of the latter would disappear.

The results obtained using  $\text{CO-O}_2\text{-NO}_2\text{-H}_2$  mixtures furnish another example of a reaction in which the probability of chain branching is small and under precise control, and support the view that the ultimate cause of ignition is a disturbance of thermal equilibrium in some favourable volume element of the reaction vessel.

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**Field equations of electric charges.** By H. T. FLINT. (*Communicated by O. W. Richardson, F.R.S.—Received 10 March 1939.*)

An argument published some time ago led to the result that the dynamical description of electric charge is subject to a certain limitation in which a length of the order of the dimensions of the electron is concerned.

This leads to a principle for the further development of equations suggested in an earlier communication leading to a field theory of electric charge. Expressions for the current density and momentum-energy tensor are deduced.

In the case where the gravitational field is negligible the equations coincide with those suggested by Proca and found to be applicable in a modern theory of nuclear forces.

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**Some observations on cosmic rays using a large randomly-operated cloud chamber.** By E. J. WILLIAMS. (*Communicated by J. Chadwick, F.R.S.—Received 13 March 1939.*)

An account is given of a large Wilson cloud chamber, about 30 cm. deep and 30 cm. diameter. Such a chamber has a sensitive time of nearly half a second, and this, together with its large effective volume, makes it a very sensitive detector of weak radiations. The results of certain cosmic-ray observations made with this chamber are described. "Counter-control" was not used, the intrinsic efficiency of the chamber ensuring, on the average, at least one cosmic-ray track for photography. In the absence of counter-control the photographs represent the true statistical distribution of different cosmic-ray events. Results for the energy distribution of cosmic-ray particles below  $3 \times 10^8$  V are thus obtained, and also some indications of the relative frequency of showers and of slow penetrating particles (mesotrons). Certain details of earlier work by E. Pickup and the writer on heavily ionizing tracks, which provided evidence for mesotrons, are also given in this paper. Some photographs are reproduced including one of associated high energy tracks indicating pair production of mesotrons.

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**Low temperature and insect activity.** By K. MELLANBY. (*Communicated by J. S. Gardiner, F.R.S.—Received 15 March 1939.*)

The "chill-coma temperature" for five species of insect belonging to three different orders was found to be considerably affected by the temperature at which they had been living. Individuals of the same species from warm conditions were immobilized at higher temperatures than those from cool.

Acclimatization with respect to the chill-coma temperature usually occurred within 20 hr. When insects were cooled below the chill-coma temperature and then returned to warm conditions, the length of time taken to recover was longer, the further the creatures were cooled below the chill-coma point. During chill-coma, insects from high temperatures did not become acclimatized to the low temperature.

Insects of the same species from warm conditions were less cold-hardy, and more easily killed by exposure to conditions below zero, than those from cool. The threshold temperature for voluntary movements was usually several degrees higher than that at which movements were possible. There was some indication that the threshold is in the region of the developmental zero for the species. Within the zone of normal activity, when an insect was transferred from one temperature to another, its rate of metabolism quickly altered to that characteristic of the new temperature. It is only outside the zone of normal activity that the rate of metabolism was greatly affected by the previous conditions.

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**A crystallographic investigation of glacier structure and the mechanism of glacier flow.** By M. F. PERUTZ and G. SELIGMAN. (*Communicated by Sir William Bragg, P.R.S.—Received 15 March 1939.*)

The mechanism of glacier flow and the transformation of firn into glacier ice were investigated on the Great Aletsch Glacier and in neighbouring regions. Samples of firn or ice were taken from the walls of crevasses and from grottoes and pits dug in the glaciers. Thin sections were cut from these samples and examined by the standard methods of optical crystallography and a study made of the size, shape and orientation of the crystals and the distribution of air-spaces between them.

During the transition of firn into glacier ice the air spaces, forming a system of intercommunicating capillaries in the firn, become reduced in number and separated from each other by compression and by the growth of the crystals. This growth is primarily effected by the refreezing of melt water retained in the capillaries.

A large percentage of the firn crystals near the surface of the *névé* lie with their principal axes at right angles to the surface of the glacier. This orientation appears to be part of the general rule whereby recrystallization of snow by melting and refreezing or by sublimation involves a reorientation of the crystals with their axes parallel to the direction of the temperature gradient.

In the course of 6-8 years the initial orientation of the crystals near the glacier surface gives way to a random distribution. The change seems to be due to the haphazard motion of single crystals or clusters of single crystals which constitute independently moving units in the mechanism of flow in the *névé* area. They are the

bearers of the "differential movement" which measurements showed to be irregular on a small scale but uniform and continuous when regarded as a whole.

Stratification bands in the *névé* form by the freezing of wet strata of firn and have no connexion with any irregularities in the differential movement. The orientation of their crystals is similar to that of lake ice. The blue bands in the glacier tongue are divided into three categories: first those which are old ice bands originally present in the *névé*, second remnants of former fissures and crevasses and third those which represent slip planes and are caused by laminar motion of large strata of ice.

Examples both of uniform plastic deformation and of laminar motion were observed in the glacier tongue. In places where the ice was subjected to shear the crystals were orientated with their basal planes parallel to the direction of the shearing force. A mechanism of plastic deformation is proposed which accounts for these findings and is based on the growth, deformation and reorientation of the crystals.

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**The rest-masses of the neutron and the neutrino.** By H. O. W. RICHARDSON. (*Communicated by Sir Arthur Eddington, F.R.S.—Received 16 March 1939.*)

The  $\beta$ -decay process of electron capture is treated as a gauge transformation which neutralizes the Coulomb field in the internal wave-equation of the hydrogen atom and inserts a uniform positive potential of about 500,000 V. The corresponding potential introduced into the external wave-equation will be  $2\frac{1}{2}$  times as large. The internal particle of the neutralized atom is identified with the neutrino and has zero mass, while the external particle is the neutron, with mass equal to that of a proton and  $2\frac{1}{2}$  electrons. The two parts of the gauge transformation correspond to the two parts of the transformation used by Eddington in the theory of the Coulombian interaction.

The same masses may be obtained by neutralizing the Coulomb fields of the proton and electron. Reasons are given for regarding the electron mass as an internal energy of electrostatic interaction between a charged particle and a comparison particle and the proton mass as the energy of the external particle of a similar system of charged and comparison particle.

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**The mean free path of electrons in polar crystals.** By H. FRÖHLICH and N. F. MOTT, F.R.S. (*Received 16 March 1939.*)

A theoretical calculation is given of the mean free path of the electrons in a polar crystal. The results obtained are compared with experiments on semi-conductors and photoconductors.

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**Note on copper-cuprous oxide photocells.** By N. F. MOTT, F.R.S. (*Received 16 March 1939.*)

The behaviour of copper-cuprous oxide photocells is interpreted in terms of a theory of the contact between a metal and a semi-conductor recently given by the author.

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**An investigation of the accuracy of König's formula for the Rayleigh disk.** By A. C. MERRINGTON and C. W. OATLEY. (*Communicated by O. W. Richardson, F.R.S.—Received 17 March 1939.*)

The original object of these experiments was to determine whether the couple acting on a Rayleigh disk suspended in a sound field in air is affected by the viscosity of the air. Since any effect due to viscosity should be relatively more important at low pressures, measurements were made of the way in which the couple varies with pressure. No definite evidence of an effect due to viscosity was obtained, but the experiments brought to light a hitherto unsuspected discrepancy between the measured values of the couple acting on the Rayleigh disk and those calculated from King's corrected version of König's formula.

A second series of experiments, in which an accuracy of about 1 part in 200 was attained, served to confirm the existence of a discrepancy between theory and experiment. Further tests showed that this discrepancy is to be attributed to the formation of vortices in the medium near the disk. It seems probable that these vortices will nearly always be present under the conditions existing in the normal use of a Rayleigh disk for acoustic measurements.

The following empirical equation is proposed to represent the relation between the couple  $G$  acting upon the disk, the density of the medium  $\rho$ , the mean-square velocity of the medium  $\bar{u}^2$ , the radius of the disk  $r$  and the angle  $\theta$  between the axis of the disk and the direction of flow of the medium:

$$G = 1.47 r^3 \rho \bar{u}^2 \sin 2\theta \left( \frac{m_1}{m_1 + m_0} \right).$$

In this equation  $m_1$  is the mass of the disk and  $m_0$  its hydrodynamic mass, which is equal to  $\frac{8}{3}\rho r^3$ . The equation has not been verified for frequencies greater than 50 cyc./sec.

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**The two-dimensional hydrodynamical theory of moving aerofoils. III.** By R. M. MORRIS. (*Communicated by G. I. Taylor, F.R.S.—Received 17 March 1939.*)

This paper follows two previous ones on the general hydrodynamical problem of the two-dimensional motion of a cylinder in inviscid incompressible fluid. The general formulae obtained in these papers are here reduced for the special case of the Joukowski aerofoil, where the coefficients assume simple finite forms. The formulae obtained are then applied to a determination of the lift, moment and centre of pressure in uniform

rectilinear motion, and also to a discussion of the dynamics of the small oscillation about such rectilinear motion. A simple condition is then obtained for the stability of this rectilinear motion, showing the effects of thickness, camber and angle of attack on this important property of the aerofoil.

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**Crystallographic studies of meteoric iron.** By J. YOUNG. (*Communicated by S. W. J. Smith, F.R.S.—Received 20 March 1939.*)

A detailed investigation of the mutual orientations of the chief constituents, kamacite and taenite, of the octahedral meteoric irons and their relationship to the Widmanstätten structure has been made.

In Cañon Diablo (part I), fifteen crystals belonging to the macroscopic Widmanstätten structure were separately examined. These showed small deviations from the twenty-four orientations of the type,

(110)-kamacite parallel to (111)-octahedrite.

[001]-kamacite inclined at  $4.1^\circ$  ( $\mu$ ) to [110]-octahedrite.

In Butler and Carlton (part II), the microscopic Widmanstätten structure was examined. The taenite crystals showed a very strongly preferred single orientation identical with that of the octahedrite, while the kamacite crystals were orientated in the same way as those in Cañon Diablo.

In a heat-treated artificial nickel-iron (part II), containing 10% of nickel the orientations of the  $\gamma$ -phase and  $\alpha$ -phase constituents were found to be similar to those of the taenite and kamacite, respectively, in the meteoric irons. The value of  $\mu$  ( $3.4^\circ$ ), however, was somewhat lower.

In all cases in which  $\mu$  could be determined with some precision the orientation of an  $\alpha$ -phase crystal lay between that found by Nishiyama and that proposed by Kurdjumow and Sachs.

An attempt is made (part III) to account for the orientations found by supposing that the  $\gamma$ - $\alpha$  transformation proceeds according to a lattice mechanism of the Nishiyama-type modified by the effect of temperature agitation. Reasons are given for supposing that the orientations for which  $\mu = 0^\circ$  and  $\mu = 5^\circ 16'$  involve high potential energies at the kamacite-taenite interface and the consequent avoidance of such orientations of the kamacite crystals.

These orientation studies give the most convincing proof that the body-centred  $\alpha$ -crystals of kamacite are the result of a precipitation from a single face-centred  $\gamma$ -lattice of uniform orientation whose axes are those of the octahedrite.

The distortion of the Widmanstätten structure was also examined (part I). It is shown that the bulk distortion of the Widmanstätten structure and the rotations of individual crystals are consistent with the effects of plastic shearing of the whole portion of meteorite examined.

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**A magnetic study of the iron-nickel-aluminium system.** By W. SUCKSMITH. (*Communicated by A. M. Tyndall, F.R.S.—Received 20 March 1939.*)

A survey of the magnetic saturation intensities of the iron-nickel-aluminium system at temperatures up to the Curie point is described, in which over eighty alloys have been examined. It is shown that each phase region exhibits characteristic features, and phase changes can in general be followed by the variation of the saturation intensity. The differences between the saturation intensities of ordered and disordered alloys is examined in various regions of the phase diagram. The more detailed examination of alloys showing special features is in progress.

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**The combustion of aromatic and alicyclic hydrocarbons. II. The ignition of aromatic hydrocarbons at high temperatures.** By J. H. BURGOYNE. (*Communicated by A. C. Egerton, Sec.R.S.—Received 20 March 1939.*)

The ignition reactions between 450 and 750° C of benzene and seven of its alkyl derivatives have been studied. The applicability of the ignition pressure-temperature relationship  $\log(p/T) = A/T + B$  is examined and it is found that  $A$  increases with temperature in the case of benzene, ethylbenzene and *n*-propylbenzene, but otherwise is constant. With the same three exceptions, the correspondence between the most ignitable and the most easily combustible (isothermally) fuel-oxygen mixtures is consistent. Dilution with nitrogen decreases the critical ignition pressure, but helium has the reverse effect. An increase in the surface-volume ratio also inhibits ignition.

It is concluded that branching chain mechanisms play an important part in the ignition of benzene and its higher mono-alkyl derivatives, but with the methylbenzenes the reaction is of a more thermal character.

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**Diamagnetic anisotropy of organic molecules.** By K. LONSDALE. (*Communicated by Sir William Bragg, P.R.S.—Received 22 March 1939.*)

The principal diamagnetic susceptibilities of single molecules of aliphatic and aromatic compounds have been measured and related, where possible, to their dimensions, electronic configuration, optical polarizabilities, magnetic birefringence and other physical properties. It is found that for long-chain compounds containing few or no multiple bonds, the maximum diamagnetic susceptibility and maximum refractivity correspond to the length of the molecule. In molecules of layer structure also containing few or no multiple bonds, the maximum diamagnetic susceptibility and maximum refractivity both lie in the mean plane of the layer, along the direction of greatest length. Both these types of compound show negative magnetic birefringence.

In aliphatic molecules containing acetate or acid groups, or chains of conjugated double and single bonds, such groups tend to be plane and show an abnormally large diamagnetic susceptibility normal to the plane of the group. This produces a magnetic anisotropy opposite in sign to that found for aliphatic layer structures containing only single bonds, but similar to that characteristic of aromatic molecules.

Aromatic molecules with aliphatic conjugated side-chains possess magnetic anisotropy greater than that which would be expected for the aromatic part of the molecule alone. For all these substances, both aliphatic and aromatic, the abnormally large diamagnetic susceptibility corresponds in direction with the minimum optical polarizability. They therefore show a positive, often large, value of the magnetic birefringence.

There is at present no theoretical treatment which accounts for diamagnetic anisotropy in any but conjugated ring structures.

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**Finite complexes in crystals: a classification and review.** By A. F. WELLS.  
(Communicated by J. D. Bernal, F.R.S.—Received 22 March 1939.)

In crystals in which bonds of more than one type occur we may distinguish aggregates of atoms within which the binding is of one kind whereas that between one aggregate and the next is of a different nature. Such groups of atoms may be finite (molecules or complex ions) or they may extend indefinitely in one or two dimensions throughout the crystal. The problem dealt with here is the enumeration and classification of finite complexes. The disadvantages of classifications based on chemical formulae and/or point symmetry are discussed, and the method adopted is to consider the number and arrangement of the atoms of one kind only. This atom will in general be electropositive with respect to the other atoms in the complex and will in general form more bonds than these. A complex  $A_mX_n$  is referred to an arrangement  $A_m$  in which the  $A$  atoms are linked together as in the original complex,  $A-A$  replacing  $A-X-A$ . The problem is thereby reduced to finding in which ways a specified number of points (atoms) may be arranged in space to form a connected system. The number of links which may meet in any point is limited to four, i.e. for  $p$  points the maximum number of links considered is  $2p$ . Within these limits and with certain other restrictions the possible systems of points and links are enumerated for values of  $p$  from 3 to 12. The second part of the paper deals with the ions and molecules (known and unknown) which may arise by placing  $X$  atoms on the links (and in the case of polyhedral complexes, on the faces) of these systems  $A_m$ .

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**Adrenaline and muscular exercise.** By F. C. COURTICE, C. G. DOUGLAS, F.R.S., and J. G. PRIESTLEY. (Received 27 March 1939.)

The effect of a single subcutaneous injection of adrenaline has been studied in two subjects during light and moderate muscular exercise. Hyperglycaemia caused by a given dose of adrenaline is less during exercise than during rest, while the concentration of excess lactic acid in the blood is much the same in the two cases. The excess lactic acid disappears from the blood practically as rapidly during exercise as during rest. The changes of respiratory quotient, when followed in detail, appear to depend on the accumulation and subsequent disappearance of excess lactic acid, and afford no evidence that there is an enhanced oxidation of carbohydrate in spite of the long-maintained rise of blood-sugar concentration.

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1 MAY 1939

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**The subjective judgment of the elastic and plastic properties of soft bodies. I. The differential thresholds for viscosities and compression moduli.** By G. W. S. BLAIR and F. M. V. COPPEN. (*Communicated by F. C. Bartlett, F.R.S.—Received 23 March 1939.*)

It is suggested that the firmness and "elasticity" (in the colloquial sense of the word) of materials which are neither true fluids nor perfectly elastic solids may be conveniently described by an equation of the type

$$\log S = \log \psi + \log \sigma - k \log t,$$

where  $S$  = stress,  $\sigma$  = strain,  $t$  = time, and  $\psi$  and  $k$  are parameters representing firmness and "elasticity" respectively.

As a preliminary to investigating the capacity of skilled craftsmen and unskilled individuals to judge firmness and "elasticity" by means of the senses, "differential thresholds" were measured for the viscosity of a true fluid and the compression modulus of an approximately elastic solid.

Technological experience, and scientific and non-scientific education do not affect the sensitivity of the subject, nor is there much improvement with practice. In the case of viscosity, a number of subjects having widely differing training hardly differed in "thresholds". With modulus, there were specific differences between subjects, but these were not simply related to training. The effects of age, sex, etc., are discussed.

Subjects can judge differences in compression modulus three times as small as those which can be distinguished for viscosity. Differences of the order of 6% in the case of modulus and 18% in the case of viscosity can be detected with some measure of certainty. The use of a single point "threshold" is, however, not recommended, although it is found that a simple equation involving two parameters fits the data obtained. This equation must be regarded as provisional and empirical.

Since the superior capacity of experts appears to depend on no greater sensory acuity for the properties investigated than that of other subjects, nor is there much improvement in this acuity with practice, it seems likely that some type of functional standard is developed which improves the capacity for recognition of rheological conditions. To this must be added the advantages of technological knowledge, making possible adequate utilization of the information obtained. It may be that in the case of the more complex rheological properties not yet investigated, these factors are of even greater importance than in the case of viscosity and compression modulus.

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**The properties of flow of liquid He II.** By J. F. ALLEN and A. D. MISENER.  
(Communicated by J. D. Cockcroft, F.R.S.—Received 27 March 1939.)

By measuring the flow through capillaries and through fine powder, the hydrodynamic properties of liquid He II have been investigated. Capillary channels varying from 1 mm. to  $1 \times 10^{-5}$  cm. in diameter were used. The flow through capillaries was found to bear no relationship to ordinary laminar or turbulent flow, although the latter conditions were approached for large capillaries at temperatures close to the  $\lambda$ -point. The effect upon the flow of length of channel was investigated with capillaries varying in length from 40 cm. to 1 mm. Classical hydrodynamic flow was observed only in the shortest capillary which corresponded essentially to an orifice. The flow through powder (tightly packed jeweller's rouge) gave results which could be interpreted according to classical hydrodynamics over the whole experimental temperature range in He II, from the  $\lambda$ -point down to  $1.15^\circ$  K.

The main features of the flow of He II are summarized below.

The dependence of the velocity of flow on pressure became less (a) as the radius of the capillary was reduced, or (b) as the capillary was lengthened, or (c) as the temperature was lowered.

In the largest capillaries at a temperature close to the  $\lambda$ -point, an approximation to laminar conditions of flow was observed.

In all capillaries at low pressures, the velocity increased with decreasing temperature, but the reverse held at higher pressures in large capillaries.

At all temperatures there was a minimum in the relation between the radius of the channel and the velocity at constant pressure. For channels smaller than  $5 \times 10^{-4}$  cm. in width, the velocity increased rapidly with decreasing channel size.

At pressures above 50 dynes/cm.<sup>2</sup> in the narrowest channels, the velocity was completely independent of pressure at all temperatures. The curve between the pressure-independent velocity and the temperature bears a strong resemblance both in magnitude and shape to that for mobile surface films of He II above the hydrostatic surface of the liquid.

In channels less than  $10^{-3}$  cm. in width, at temperatures close to the  $\lambda$ -point, and at low pressures, the flow became laminar with evidence of an exceedingly small viscosity.

For flow through tightly packed fine powder, the observed viscosity appeared equal in magnitude and temperature variation to that observed in the narrowest smooth channels.



These results have been interpreted in terms of two types of flow occurring together in the liquid; one, a surface flow along the walls of the capillary, has an extremely small viscosity that varies from  $1 \times 10^{-8}$  c.g.s. units at  $2.177^\circ \text{K}$ , near the  $\lambda$ -point, to less than  $1 \times 10^{-10}$  c.g.s. units at  $1.15^\circ \text{K}$ , and appears to approach a value of zero at the absolute zero. The other, predominant in large capillaries, has a viscosity of the order of  $10^{-4}$  c.g.s. units which increases with decreasing temperature, and has a value just below the  $\lambda$ -point probably not much different from that of He I.

Several experiments have been made with the object of detecting possible thermal effects associated with the flow of He II. Although one experiment yielded results which suggest that a temperature difference is set up between the ends of a narrow channel in which liquid is flowing, the results cannot be considered conclusive.

**A theory of electric polarization, electro-optical Kerr effect and electric saturation in liquids and solutions.** By A. PIEKARA. (*Communicated by R. H. Fowler, F.R.S.—Received 28 March 1939.*)

This paper makes calculations of the electrical polarizability and of the Kerr effect in certain types of liquids and solutions formed of or containing polar molecules. The calculations are made on the basis that, besides the external electrical field  $E$  acting on each dipole, two constraining electrical fields act:

(1) A field of potential energy  $-W_1 \cos \theta$  due to the general directional field of all the surrounding molecules, the direction  $\theta_1 = 0$  of the direction of equilibrium taking all possible directions in space when we take averages over the whole liquid.

(2) A field of potential energy  $W_2 \cos \theta_2$  due to a single neighbour which tends to take up an antiparallel orientation. It is suggested that this model should be applicable to nitrobenzene or its solution.

On this basis approximate formulae are obtained for the reducing factor  $R$  for the electric polarization  $P$  per molecule defined by

$$P_{\text{liquid}}^{\text{dipole}} = P_{\text{gas}}^{\text{dipole}} R,$$

and compared most successfully with observations of  $R$  for nitrobenzene in hexane at  $25^\circ$  and various concentrations. Similar calculations are then made for a similar coefficient in the Kerr effect, and compared satisfactorily with experiment. The calculations are then extended to higher powers of  $\mu E/kT$ , and enable certain features of the nature of the saturation effects for strong fields to be understood.

**The compressibilities and expansion coefficients of gases at low pressures, and their relation to molecular volume.** By J. B. M. COPPOCK. (*Communicated by J. Kenyon, F.R.S.—Received 29 March 1939.*)

The compressibilities and expansion coefficients of several gases at low pressures have been calculated from the Beattie-Bridgeman equation of state. It is shown for

non-polar gases that a linear relation exists between these physical constants and the corresponding molecular volumes. The latter have been calculated from molecular radii based on the Sidgwick-Pauling additivity rule. The behaviour of certain hydrocarbons is discussed in the light of their spatial structure. It is suggested from the relation developed that the nearest distance of approach of non-polar molecules at low pressures is dependent on their size and is not a uniform amount as represented by an envelope of about 0.5 Å thick.

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### **X-ray investigation of the structure transition of methane at the $\lambda$ point.**

By A. SCHALLAMACH. (*Communicated by Sir William Bragg, P.R.S.—Received 30 March 1939.*)

An X-ray investigation of methane made in the neighbourhood of the  $\lambda$  point shows that the structure when observed well below and above the transition point is a face-centred cube. This agrees with the results obtained by previous observers. The X-ray examination shows that near the  $\lambda$  point there are spacings additional to those belonging to the face-centred cube, and these must be ascribed to certain intermediate structures existing only in the transformation region. It is pointed out that the presence of such structures must necessarily influence the specific heat in the transition range.

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### **Luminescence of mercury vapour along an $\alpha$ -ray pencil, and transfer of nitrogen excitation to mercury.** By A. LUYCKX. (*Communicated by Lord Rayleigh, F.R.S.—Received 3 April 1939.*)

It is shown that mercury vapour luminescence phenomena similar to the optically excited fluorescence phenomena, can be excited by  $\alpha$ -rays excitation in presence of nitrogen. The green luminescence renders visible an  $\alpha$ -ray pencil by light emission, this new method is quite different from the well-known expansion method of C. T. R. Wilson. However, in this case no individual track was seen. The emission spectrum of this luminescence consists of a visible mercury triplet (5461, 4358, 4046) a continuum having a maximum near 4850 Å and the resonance line 2537 Å.

Persistent phenomena were observed. The spectra and persistent phenomena are similar to the fluorescent phenomena.

No luminescence can be seen in absence of nitrogen. In a primary process the  $\alpha$ -rays excite nitrogen molecules which transfer their energy to mercury by collisions of the second kind. The green luminescence is extinguished by small quantities of air, while hydrogen has no quenching effect. This survival of the glow to addition of large quantities of hydrogen brought us to assume that this luminescence is a sort of "wing effect" due to the transfer to diatomic mercury molecules of the energy of the nitrogen molecules excited by  $\alpha$ -rays.

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18 MAY 1939

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**Variations of ionization in the  $F_2$  region of the ionosphere. I. Meteorological associations.** By J. BANNON, A. J. HIGGS, D. F. MARTYN and G. H. MUNCE.  
(Communicated by T. H. Laby, F.R.S.—Received 4 April 1939.)

A study has been made of the day-to-day variations in the noon values of the maximum electron density in the  $F_2$  region of the ionosphere, at two observing stations near Sydney and Canberra. The small number of days on which these values are affected by association with magnetic storms are excluded from the analysis. Large day-to-day fluctuations, amounting sometimes to 50 %, remain at each station. It is found that these fluctuations are associated with meteorological changes observed at the ground. Over 19 months of daily observations it is found that on days when the region between lat.  $29^\circ$  S. and lat.  $36^\circ$  S., and long.  $140^\circ$  E. and long.  $156^\circ$  E. is free from "frontal" conditions, the values of  $F_2$  ionization are higher on the average by 6 % near Sydney and 11 % near Canberra than on other days. A similar difference is found in the averages for each month except in August 1937 and July and August 1938 when a small negative difference occurs at each station. The difference is greatest in the equinoctial months when it amounts to about 20 % for Stromlo. It is found that while the values of ionization density measured at Sydney and Canberra are usually equal, there is a strong tendency for the Canberra values to be lower than the Sydney values on "frontal" days. This result is considered surprising, since the observing stations are only 250 km. apart.

The theoretical difficulties in the way of an explanation of the association here found between  $F_2$  ionization and meteorological conditions are discussed.

It is pointed out that the results suggest that current views concerning the seasonal variation of  $F_2$  ionization in various regions of the earth may require revision. The current view regards the  $F_2$  region ionization as normally influenced solely by the

intensity of solar radiation, thus depending in magnitude solely on latitude. The results here presented suggest that local climatological factors may exert a profound influence on the magnitude, and on the seasonal and diurnal variations of  $F_2$  ionization.

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**The excitation of  $\gamma$ -radiation in processes of proton capture.** By S. C. CURRAN and J. E. STROTHERS. (*Communicated by W. L. Bragg, F.R.S.—Received 5 April 1939.*)

The excitation functions for the  $\gamma$ -radiations emitted by the elements deuterium, lithium, sodium, magnesium, phosphorus and chlorine under proton bombardment have been determined for energies of the incident protons from 0 to  $10^8$  eV.

Resonance peaks, hitherto unobserved, have been found in the excitation curves for incident protons of the energies given below.

$^{23}\text{Na}$  : 425, 525, 590, 690, 755,  $875 \times 10^3$  eV.

$^{25}\text{Mg}$  : 180, 410, 480, 575,  $825 \times 10^3$  eV.

$^{26}\text{Mg}$  : 580, 680,  $1000 \times 10^3$  eV.

$^{31}\text{P}$  : 460, 580, 700,  $950 \times 10^3$  eV.

$^{37}\text{Cl}$  : 650, 800,  $1000 \times 10^3$  eV.

In the case of  $^7\text{Li}$  no evidence of any resonance peak other than that at 440 kV could be obtained. No capture  $\gamma$ -radiation could be detected during the bombardment of  $^6\text{Li}$  and deuterium with protons of energies up to  $10^8$  eV.

The yield of  $\gamma$ -radiation was measured in the case of  $^{25}\text{Mg}$  and  $^{26}\text{Mg}$ , the values obtained being  $3.0 \times 10^{-10}$  quanta per proton at 825 kV ( $\text{Mg}^{25}$ ) and  $2.1 \times 10^{-10}$  for  $\text{Mg}^{26}$  at 680 kV.

The energy of the capture  $\gamma$ -radiation was measured in each case from observations of the end-points of the absorption curves which were obtained by placing aluminium sheets between the pair of Geiger-Müller counters used for the detection of the  $\gamma$ -rays.

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**The energy levels of a rotating vibrator.** By I. SANDEMAN. (*Communicated by H. S. Allen, F.R.S.—Received 6 April 1939.*)

The theoretical work of the late J. L. Dunham based on the rotating vibrator model for the diatomic molecule is extended and simplified. While the simplifications introduced are necessarily not very far-reaching, they mean an appreciable gain in the rapidity with which the molecular constants can be calculated by Dunham's method from spectral data.

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**Classical theory of mesons.** By H. J. BHABHA. (*Communicated by P. A. M. Dirac, F.R.S.—Received 11 April 1939.*)

The vector equations for the meson field and their associated energy tensor are taken as exact, and the meson field quantities are taken as commuting classical variables. A self-consistent classical scheme is developed for treating the meson field and point neutrons (or protons) moving along classical world lines. The scheme takes account of the reaction of the emitted meson field on the motion of the neutron. The mass of the neutron (or proton) which occurs in nuclear phenomena is shown not to be the real mass but less than it by about ten million volts. For high energy phenomena the real mass counts. Formulae are given for the scattering of mesons by neutrons which are valid to energies comparable with the mass of the neutron, and supersede the previous formulæ. It is shown that classically the reaction of the emitted radiation is not important till energies of ten times the neutron mass, and hence that the quantized meson theory which neglects this is valid up to about these energies. It is concluded that the exact quantized theory is correct with the above limitation, but that the methods of perturbation theory break down in this case and cannot be applied to it.

**An interpretation of the relativity transformations of the electromagnetic field for small velocities.** By E. G. CULLWICK. (*Communicated by Sir Frank Smith, F.R.S.—Received 14 April 1939.*)

It is shown that the relativity transformations of the electromagnetic field can be obtained by a "moving field" theory in which the chief postulates are as follows:

(a) The apparent state of electrification of an isolated moving magnet or current circuit is to be independent of its uniform linear motion.

(b) If a body is moving with uniform velocity  $v$  relative to the source of a magnetic field  $B$ , it is subjected to an electric field of value  $\mathcal{E} = v \times B$ .

Conversely, if the source of a magnetic field  $B$  is moving with velocity  $v$  relative to a body, then the body is subjected to an electric field given by  $\mathcal{E} = B \times v$ .

(c) If a body is moving with uniform velocity  $v$  relative to the source of an electrostatic field  $\mathcal{E}_s$ , it is subjected to a magnetic field given by  $B = (\mathcal{E}_s \times v)/c^2$ .

Conversely, if the source of an electrostatic field  $\mathcal{E}_s$  is moving with velocity  $v$  relative to a body, then the body is subjected to a magnetic field given by  $B = (v \times \mathcal{E}_s)/c^2$ . The experiment of H. A. and M. Wilson (1913, *Proc. Roy. Soc. A*, 79, 99) on a rotating magnetic insulator is then examined. It is shown that the moving-field theory, extended to include the case of rotation, gives a simple derivation of the result verified by the experiment, and that the usual "magneto-polarization" modification of the Maxwell-Lorentz theory, which is claimed to be consistent with the experiment, fails in logical consistency.

It is therefore suggested that the logical electromagnetic concomitant of the restricted relativity theory is not the Maxwell-Lorentz theory, in which the apparent state of electrification of an isolated magnet depends upon its velocity relative to the observer, but the moving-field theory in which the state of electrification of an isolated magnet is postulated to be independent of its uniform motion. To this theory may be added the usual relativity modifications of electrostatic and magnetostatic fields where high velocities are concerned.

**Fine structure of the Raman lines of carbon tetrachloride.** By A. C. MENZIES. (*Communicated by R. Whiddington, F.R.S.—Received 18 April 1939.*)

The intensities in the fine structure of the Raman line corresponding to the total symmetric vibration in  $\text{CCl}_4$  have been examined quantitatively, and are consistent with a chlorine isotope explanation, since the analysis leads to an abundance ratio of about 3.15, in good agreement with Aston's value from mass spectra.

The total-symmetric vibration line ( $992 \text{ cm.}^{-1}$ ) in benzene, and the components of the  $459 \text{ cm.}^{-1}$  line in  $\text{CCl}_4$ , are considerably wider than the exciting line.

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**The mobility of alkali ions in gases. I. The attachment of water molecules to alkali ions in gases.** By R. J. MUNSON and A. M. TYNDALL, F.R.S. (*Received 18 April 1939.*)

The formation of clusters by the attachment of water molecules to alkali ions has been studied by measurements of the mobilities of these ions in the inert gases when small concentrations of water vapour are also present. In helium and neon the mobility of the clusters passes through a minimum as the mass of the alkali ion is increased. Thus the sodium clustered ion has the least and the caesium clustered ion the greatest mobility in the series, which suggests a decrease in the equilibrium number of attached molecules with increasing radius of the alkali atomic ion. In argon, krypton and xenon an *upper limit* of six molecules can be assigned to the number of water molecules in the cluster and the true number is probably less than six.

The process of clustering has been followed by passing the ions through an ageing field and then measuring their mobility. Only two groups of ions were found, one consisting of clustered ions and the other of unclustered, their relative numbers depending on the time spent in the ageing field and the concentration of water vapour in the gas. By varying the conditions the fraction of clustered ions could be varied from unity at very low ageing fields to zero at high fields. These results follow if a three body collision between an ion, a gas molecule and a water molecule is necessary for the attachment of the first water molecule but that having attached the first molecule the cluster quickly builds up to a given size. Experimental work at different gas pressures supports this view.

With increasing field the energy of agitation of an ion must increase in association with its velocity of drift in the direction of the field. Since this is equivalent to a local higher temperature one would then expect a reduction in the number of water molecules that an ion can retain in attachment. It is found, in agreement with this view, that with increase of field the mobility of the clustered ion at a given gas pressure increases and gradually approaches that of the unclustered ion.

The paper contains a table of mobility values for all the alkali ions in all the inert gases, together with those for most of the water clusters of maximum size.

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**The mobility of alkali ions in gases. II. The attachment of inert gas atoms to alkali ions.** By K. HOSELITZ and R. J. MUNSON. (*Communicated by A. M. Tyndall, F.R.S.—Received 18 April 1939.*)

Evidence of the attachment of inert gas atoms to a lithium ion has been obtained in all the inert gases. The condition necessary for attachment is that the energy of thermal agitation of the ion and of gas atoms through which it moves must not exceed a given value which depends upon the dissociation energy of the molecule so formed. At low values of field/pressure ( $E/p$ ), when the ion is in thermal equilibrium with the surrounding gas, an appreciable fraction of the Li ions in He are of the form  $\text{Li}^+, n(\text{He})$  at  $20^\circ \text{K}$ ; the fraction is much smaller at  $90^\circ \text{K}$  and zero at room temperature. The value of  $n$  is not more than two and more probably nearer unity. In neon practically all the ions show attachment at  $90^\circ \text{K}$  and none at  $195^\circ \text{K}$ . The corresponding temperature limits in argon are 195 and  $290^\circ \text{K}$ . In Xe and Kr every ion collects at least one and probably two gas atoms at room temperature. In some cases there is evidence that  $n$  increases as the temperature is reduced. On the other hand, potassium ions collect no gas atoms in argon at  $195^\circ \text{K}$ , doubtless because the dissociation energy must be less than for lithium ions owing to the larger size of the potassium ion.

If the value of  $E/p$  is raised, these molecular ions tend to disappear owing to the increased thermal energy of the ion which is associated with its drift in the field. From estimates made of the fraction of ions which are molecular at a given temperature, upper and lower limits have been set to the values of the dissociation energy of a lithium ion and gas atom. It is found that the value increases with the mass of the gas atom, i.e. with its polarizability; it ranges from about 0.07 eV in helium to 0.33 in krypton and is  $>0.42$  in xenon.

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**The mobility of alkali ions in gases. III. The mobility of alkali ions in water vapour.** By R. J. MUNSON. (*Communicated by A. M. Tyndall, F.R.S.—Received 18 April 1939.*)

Determinations of the mobility of alkali ions in water vapour show that at low values of field/pressure ( $E/p$ ) the mobility decreases slightly with increase in mass of the alkali atom. This sequence changes entirely when  $E/p$  is considerably increased. The latter result is qualitatively explained by the gradual break up of the cluster of molecules attached to the ion as its kinetic energy increases; the variation of the mobility with increasing  $E/p$  will depend upon the energy of dissociation of a water molecule from an alkali ion and upon the fractional change in the mass of an ion caused by the loss of a water molecule from the cluster, both of which depend upon the nature of the alkali ion under consideration. The results at low  $E/p$  are briefly discussed in comparison with the behaviour of alkali ions in liquids and of water clustered ions in gases.

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**Theory of electrical breakdown in ionic crystals. Part 2.** By H. FRÖHLICH.  
(Communicated by N. F. Mott, F.R.S.—Received 24 April 1939.)

Following the ideas on electrical breakdown developed in a previous paper, a formula for the breakdown field strength is derived which holds for any kind of polar crystal. This formula makes possible the quantitative calculation of the breakdown field from the optical properties of the dielectric, the dielectric constant and from the molecular volume. The breakdown field  $F$  has been calculated for the alkali halides for quartz and for mica. Satisfactory agreement with experimental values, when they exist, is obtained. It follows from the theory that  $F$  should be practically independent of temperature in the case of silicates, in agreement with experiment. For the alkali halides, however,  $F$  should increase with increasing temperature. For films thinner than about  $10^{-5}$  cm.,  $F$  should increase with decreasing thickness.

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**Tides in oceans bounded by meridians. IV. Series solutions in terms of angular width of ocean: semidiurnal tides in narrow oceans.** By A. T. DOODSON, F.R.S. (Received 24 April 1939.)

The first three parts of this series of memoirs (Proudman 1936; Doodson 1936, 1938) have been concerned with the tides in an ocean bounded by a complete meridian, and the main issue was that of the variation of the diurnal and semidiurnal tides with the variation of the ocean. The methods used in the first three parts are derived from a general method due to Proudman, which will in due course be applied to other oceans.

Other methods of attacking the problems have been exploited. One line of investigation is to consider a very narrow ocean and to attempt to develop the solution stage by stage in terms of series of powers of the angular width of the ocean. The method has one outstanding feature in that it does not require the solution of a number of simultaneous equations, but it suffers from the disadvantage that it is only applicable to narrow oceans. The solution is illustrated for the semidiurnal tide ( $K_2$ ) in an ocean  $30^\circ$  wide.

Special consideration is given to the elucidation of the motion in very narrow oceans and to the results indicated by analytical methods for certain special depths.

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**Tides in oceans bounded by meridians. V. Solutions by use of finite differences: semidiurnal tides.** By A. T. DOODSON, F.R.S. (Received 24 April 1939.)

The methods used in this memoir are essentially very different in character from those developed in the first four parts (Proudman 1936; Doodson 1936, 1938, 1939) and in one sense they are experimental as the methods of finite differences on such a large scale have never been exploited in connexion with tides in oceans. The great

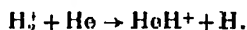


difficulties involved in the use of the normal mathematical functions, due to slow convergence of series, even for simplified oceans, appears to set a limit to their use, whereas finite differences can find application, or should be usable, for any types of variations of ocean depths and contours, so that undoubtedly there is a large field of use. In this memoir the methods have been successfully applied to the evaluation of the semidiurnal tide ( $K_2$ ) in oceans of constant depth up to  $90^\circ$  wide, five cases being evaluated and illustrated. Though it is not expected that the solutions are of great accuracy, yet the variations in the character of the solutions are probably quite genuine, and offer a trustworthy guide to the relation between the dynamical response of the tidal motion and the width of the ocean.

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**The formation of  $\text{HeH}^+$  molecules.** By F. L. ARNOT and M. B. M'EWEN.  
(Communicated by H. S. Allen, F.R.S.—Received 24 April 1939.)

An investigation of the formation of the singly-charged helium molecule ( $\text{HeH}^+$ ) has been made by means of a mass spectrograph. It is found that the molecule is formed by a collision between a singly-charged hydrogen molecule and a normal helium atom in which the hydrogen molecule is dissociated. The process is



For this process to be energetically possible the colliding particles must have kinetic energy in excess of the difference between the energy of dissociation of  $\text{H}_2^+$  (2.6 eV) and that of  $\text{HeH}^+$ , which Coulson and Duncanson find from a wave-mechanical treatment to be about 1.5 eV. The kinetic energy of the  $\text{H}_2^+$  ion must therefore be greater than 1.1 eV. The ionization potential of the  $\text{HeH}$  molecule is found to be 12 V. No evidence of the existence of a  $\text{HeH}_2$  molecule was found.

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**Evidence of resistance to tumour growth in the offspring of immunized rats.** By S. RUSS and G. M. SCOTT. (Communicated by R. A. Fisher, F.R.S.—Received 24 April 1939.)

If the results of these groups of experiments are considered together, the average volume of the Jensen's rat sarcoma tumours in 385 rats, the offspring of immunized parents, is just under half of the controls 17–21 days after inoculation when most carefully controlled, and just over half the volume when only the male parent has been immunized.

The number of disappearing tumours was much greater in the offspring of the immunized rats, 42.0 % disappearing compared with 10.0 % in the controls.

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**Distribution of charge and potential in an electrolyte bounded by two plane infinite parallel plates.** By L. ROSENHEAD. (*Communicated by W. C. M. Lewis, F.R.S.—Received 26 April 1939.*)

In 1925 Zocher noticed that certain colloid substances separated out in horizontal layers at the bottom of the vessels which contained them. He noticed also that the distance between consecutive layers diminished as the number of layers above the point of observation increased. A theoretical explanation of this phenomenon was put forward in 1938 based on certain assumptions and a dependent approximate mathematical evaluation of the force between the layers. The mathematical evaluation of the force is open to criticism.

The present investigation is based upon assumptions which are simpler than those put forward by Zocher, and on an exact evaluation of the electrostatic charge and force associated with the presence of an electrolyte between two plane infinite parallel plates. The force is evaluated on the basis of the Gouy theory, the plates being separated by approximately  $3 \times 10^{-5}$  cm.

The effects of three alternative assumptions are investigated and it is found that only one of them leads to results which are in agreement with observed experimental facts. The electrokinetic potential difference is found to vary with the concentration of the electrolyte, the range of values consistent with experimental results being 5–22 mV. In the Zocher investigation the electrokinetic potential difference emerged as a consequence of the assumptions and was found to be 88 mV.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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6 JUNE 1939

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**Dielectric loss due to polar molecules in solid paraffin wax.** By D. R. PELMORE. (*Communicated by E. V. Appleton, F.R.S. - Received 27 April 1939.*)

This paper describes an extension of the work of Jackson and Sillars who investigated the causes of dielectric loss in systems consisting of paraffin wax and an aliphatic ester (W. Jackson, 1935, *Proc. Roy. Soc. A*, 150, 197; R. W. Sillars, 1938, *Proc. Roy. Soc. A*, 169, 66). It confirms their conclusions that the loss in these systems is due to the rotation of the polar molecules and can be interpreted in terms of the Debye theory.

The effect of different paraffin waxes has been studied and a suggestion is made that the dielectric relaxation time of these systems depends mainly upon the lengths of the carbon chains of the molecules of the shorter component, a conclusion borne out very strongly by the comparison of the behaviour of ethyl stearate and spermaceti in hexadecane as compared with their behaviour in a natural wax whose mean chain length was approximately 26.

Experiments were made with esters containing two ester groups in each molecule, and in agreement with expectations it was found that the dielectric loss was positive or negligible according to the relative sense of the two polar groups: when these were arranged so that the dipole components transverse to the chain had opposite sense, no dielectric loss was observed. On the other hand, when the dipole components had the same sense the dielectric loss had a maximum value (at any given temperature) for a particular frequency, and the relaxation time corresponded closely with that anticipated from the chain length.

This appears to confirm the conclusion that the loss is due to the rigid rotation of the carbon chains about their long axes, and also to indicate that chain compounds

containing polar groups should be most effective as components of low-loss dielectrics prepared for practical use, when the molecules contain an even number of polar groups so arranged that one-half of the dipole components transverse to the chain have opposite sense to the remaining half.

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**The lower limiting crystallite size and internal strains in some cold-worked metals.** By W. A. WOOD. (*Communicated by G. W. C. Kaye, F.R.S.—Received 27 April 1939.*)

An X-ray examination has been made of the changes in crystalline structure during the progressive cold-working of pure copper, silver, nickel, aluminium, molybdenum and iron. It is shown that the grains are dispersed into a fundamental unit (crystallite) characterized by a lower limiting size ranging from  $10^{-6}$  cm. for aluminium to  $0.7 \times 10^{-5}$  cm. for copper. This size is deduced from the broadening of appropriate diffraction lines. It is further shown, in the general case of copper, that on continued cold-working the diffraction lines broaden to a maximum and then diminish in width to a definite value, the two processes alternating on further working. At the same time, the lattice dimensions of the crystallites change, having an expanded value when the line is most diffuse and a contracted value when the line-breadth decreases. These changes are measured and indicate that the condition of the cold-worked metal is marked by two extremes, (i) a comparatively stable state with a contracted lattice and minimum line-broadening giving the lower limiting crystallite size, and (ii) a less stable state characterized by an expanded lattice and an abnormally diffuse line-breadth which represents the extent and nature of the distortion transmitted to and retained by the metallic lattice during continued deformation.

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**Diffraction of X-rays by crystals at elevated temperatures.** By G. D. PRESTON, M.A. (*Communicated by C. H. Desch, F.R.S.—Received 28 April 1939.*)

The paper contains an account of the investigation of certain diffuse spots which occur in Laue photographs of single crystals of aluminium, rocksalt and periclase. The diffuse spots are not part of the normal Laue pattern, and although their presence has been reported by other workers it now appears that their intensity is greatly increased when photographs are taken with the crystal at about  $500^{\circ}$  C. An analysis of the photographs of aluminium shows that the spots can be accounted for by the presence in the crystal of periodic disturbances which are assumed to be the thermal vibrations of the lattice. As a result of these vibrations the crystal can be regarded as being broken up into groups of atoms which produce diffraction maxima. It is suggested that in aluminium these groups consist of an atom and its twelve neighbours, and that one group differs from another by having a slightly larger or smaller interatomic distance, or by being inclined at a small angle to the direction of crystal axes.

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**The absorption spectrum of nitrosyl chloride.** By C. F. GOODEVE and S. KATZ. (*Communicated by S. Sugden, F.R.S.—Received 28 April 1939.*)

The extinction coefficient curve of nitrosyl chloride has been measured in the visible and ultra-violet and eleven bands have been observed. Four of these in the ultra-violet and blue have been attributed to transitions to upper potential energy curves of the repulsion type. The remaining bands in the green and yellow are of the type found for transitions to vibration levels in a stable or partly stable excited state. This excited state appears to arise from a hitherto unknown level of the NO molecule. These latter bands showed no fine structure and were nearly symmetrical. A tentative assignment which is in accordance with all the observations, including studies of the effect of temperature, has been put forward.

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**Resonance phenomena in the scattering of  $\alpha$ -particles by some light nuclei.** By S. DEVONS. (*Communicated by W. L. Bragg, F.R.S.—Received 1 May 1939.*)

The scattering of  $\alpha$ -particles at  $90^\circ$  has been investigated for the elements carbon, nitrogen, oxygen, fluorine, neon and argon. The scattering substance was in the form of a gas, and the scattered particles were detected by means of an annular chamber and linear amplifier.

Resonances in the scattering have been found for  $\alpha$ -particle energies as follows: carbon, 5.7 MeV; nitrogen, 4.6 MeV, 5.2 MeV; oxygen, 5.8 MeV; fluorine, 3.5 MeV, 4.7 MeV.

The results are discussed with relation to the interpretation of scattering on the "many-body" theory of the nucleus and also in connexion with other experimental data on scattering and disintegration. The results are not of a very high accuracy, owing to low intensity and resolution of the  $\alpha$ -particle sources used.

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**A new method for studying the adsorption of gases at very low pressures and the properties of adsorbed films of oxygen on tungsten.** By J. L. MORRISON and J. K. ROBERTS. (*Communicated by E. K. Rideal, F.R.S.—Received 2 May 1939.*)

The basis of the method is the fact that the accommodation coefficient of neon with a tungsten surface is extremely sensitive to the presence of adsorbed films. Oxygen contained in a large bulb at a known partial pressure flows first through a long fine capillary tube and then through wider tubes, in which the main resistance to flow arises from the presence of the neon, to charcoal tubes immersed in liquid air, where it is removed. The wire on which the adsorption takes place is situated in one of these wider tubes, and the pressure in its neighbourhood can be calculated accurately from the kinetic theory of gases. The conditions obtaining at the surface of the charcoal have been investigated. Pressures of oxygen from about  $2 \times 10^{-9}$  to about  $7.5 \times 10^{-6}$  mm. of mercury can be obtained.

At room temperature there is a film of oxygen which is stable at the lowest pressures. Above this film is another, the population of which depends on the pressure. The isotherm for this upper film has been determined, and it has been shown that the mean life on the surface of a particle in it is not less than about two seconds. The lower film is itself composite; part of it evaporates at about 1100° K and the rest not until above 1700° K. The less stable part of this lower film is attributed to molecules adsorbed in the gaps in the immobile more stable part.

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**The kinetics of the formation of oxygen films on tungsten.** By J. L. MORRISON and J. K. ROBERTS. (*Communicated by E. K. Rideal, F.R.S.*—*Received 2 May 1939.*)

The method described in the preceding paper has been applied to the study of the kinetics of the formation of oxygen films. If  $\theta$  is the fraction of the sites on the surface which are occupied by adsorbed oxygen at time  $t$ , it has been shown that at constant pressure the variation of  $d\theta/dt$  with  $\theta$  is what would be expected for the system to which oxygen has been shown to belong in the preceding paper, i.e. a stable film with a less stable and presumably mobile film above it. The fact that the particles in this upper layer can migrate and thus have an increased opportunity of finding a suitable vacant place where they can be adsorbed into the stable film affects markedly this relation between  $d\theta/dt$  and  $\theta$ .

The relation between the accommodation coefficient  $\alpha$  and  $\theta$  is also discussed.

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**The genetical analysis of a sex-limited character in *Drosophila melanogaster* and its bearing on the evolution of secondary sexual characteristics.** By C. GORDON and F. GORDON. (*Communicated by L. Hogben, F.R.S.*—*Received 4 May 1939.*)

By selection and inbreeding of *Drosophila melanogaster*, a sex-limited palp coloration difference confined to the female can be established. Palps of females belonging to such stocks are dark brown in contradistinction to the pale palps of the male. Both sexes transmit the genetic basis of the character. In normal stocks of which the palp coloration of the female is relatively pale, there is a detectable difference between the colour of the female palp and that of the male. We may therefore regard palp coloration as an incipient secondary sexual characteristic throughout the species. The scope of this communication is the genetical analysis of the character and a discussion of its bearing on the evolution of secondary sexual characteristics.

For the analysis of its genetic basis a phenotypic index based on arbitrary numerical symbols for different grades of intensity has been used in this investigation. By a modification of the technique of "marked" chromosomes the contribution of individual chromosomes can be investigated. Brown palp stocks have been crossed with

*S/Cy*, *D/+* and with Oregon wild type. The result of such matings shows that genes which contribute to the brown palp effect are not appreciably represented in the X-chromosomes of any of the stocks examined. They are to be found on both the second and the third chromosomes, and to be equally concentrated on the second chromosome of brown palp or other chromosomes. The absence of the Y-chromosome in XO males did not result in the appearance of brown palp. The presence of a Y-chromosome in XXY females did not result in the suppression of the character. Hence the Y-chromosome does not contribute to the suppression of the character in the male. Exhibition depends on the same type of balance that determines female sexuality and depends on the equivalent of the hormone balance. Triploid stocks with autosomes predominantly from the brown palp stock were established. Triploid intersexes obtained (2X/3A) were maximally pale, i.e. male in type. (It is suggested that palp coloration might be used as an index of femaleness for the localization of sex factors.)

The relative importance of natural selection and of sexual selection in establishing secondary sexual characteristics is discussed in relation to the genetic basis of brown palp and to other genetic types of sex-limited characters.

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**On non-commuting numbers and some applications.** By S. R. MILNER, F.R.S. (*Received 4 May 1939.*)

A class of numbers, additional to the arithmetic class, is required to express changes effected in the order of the terms in an array. Its members are readily recognizable as numbers when a suitable notation and operation rules are devised for them. From these non-commuting numbers special "orthogonal" sets of related numbers can be selected which have interesting applications to four-dimensional geometry. A study of these sets brings out new results in the theories of quaternions and of rotation in four dimensions, and throws further light on the nature of the "locked" rotations and "spinors" employed in wave theory, and on Eddington's *E*-numbers.

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**The quantitative study of populations in the Lepidoptera. I.** By W. H. DOWDESWELL, R. A. FISHER, F.R.S., and E. B. FORD. (*Received 5 May 1939.*)

The numerical density of isolated populations is best estimated by marking, releasing and recapturing a number of specimens. A colony of the butterfly *Polyommatus icarus* Rott. on the island of Tean (Isles of Scilly) was studied by this method, an attempt being made to analyse the nature of any alterations in its numbers over a period of time.

A cellulose paint, recommended to us by Mr G. A. Brett, proved entirely satisfactory for marking the specimens. This seals the scales on to the wing membrane, and dries in a few seconds. It is then permanent and waterproof and causes no

damage to the insect. By the use of different colours and positions for the marks, the date of first capture, and of each subsequent recapture could be determined for any specimen.

The island could be rather sharply divided into areas which this species inhabits and from which it is absent. It was possible to show that no significant amount of migration took place to or from the colony as a whole. Consequently, additions to and losses from the existing population were attributable to emergence and to death respectively. Analysis of the data obtained showed that from 26 August to 8 September 1938, about 450-500 imagines died in all, including about 100 which emerged during that period, and that the population decreased progressively from approximately 350 individuals to almost nothing.

The present study has therefore established that the numerical density of even a rapidly changing population of Lepidoptera can be estimated and the main causes of the change distinguished.

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**Thermal diffusion in mixtures of the inert gases.** By B. E. ATKINS, R. E. BASTICK and T. L. IBBS. (*Communicated by M. L. E. Oliphant, F.R.S.—Received 8 May 1939.*)

Measurements of thermal separation have been made in all the binary mixtures of helium, neon, argon, krypton and xenon; using the Shakespear hot-wire method of gas analysis. By applying Chapman's theory the repulsive force index  $S_{12}$  of the intermolecular field has been obtained for each pair. The results show a wide and orderly range of molecular behaviour, the values of  $S_{12}$  extending from 11.4 for helium-neon to 5.2 for krypton-xenon. In all cases there is a decrease in the value of  $S_{12}$  as a heavier molecule is substituted for a lighter one in a mixture. The successive mono-atomic molecules thus become "softer" as their mass increases, i.e. their behaviour becomes less like that of rigid elastic spheres. This conclusion is in general agreement with the information given by viscosity on the molecular fields of the single gases. A comparison with Harrison's thermal diffusion value of  $S_{12}$  for helium-radon, obtained by using radioactive methods of measurement, shows that the massive radon molecule takes its proper place in the sequence as the "softest" of the group.

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**The photosynthesis of carbohydrates from hydrated carbon dioxide.** By E. C. C. BALY, F.R.S. (*Received 12 May 1939.*)

When a surface of pure nickel oxide containing thorium oxide in the molecular ratio of  $1\text{ThO}_3 : 24\text{NiO}$  is irradiated in the presence of water and carbon dioxide a carbohydrate is photosynthesized. This substance is unstable and changes slowly at room temperature and rapidly at  $60^\circ$  into a second carbohydrate which can be hydrolysed by taka-diastase to a reducing sugar and is probably, therefore, a type of starch.

The best method of preparing a surface of nickel oxide containing thorium oxide is by adsorption on purified kieselguhr. The results obtained with the supported oxides



were fully confirmed with the unsupported oxides, but this method was found to be less satisfactory.

Full details are given of the preparation of the supported and unsupported oxides and it is necessary that these be rigidly adhered to, since the presence of impurities renders the surface photosynthetically inactive.

The presence of thorium oxide in the molecular ratio of  $1\text{ThO}_2 : 24\text{NiO}$  is essential, since a surface of pure nickel oxide was proved to be inactive.

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**Recovery heat in muscle.** By A. V. HILL, Sec.R.S. (*Received 11 May 1939.*)

The recovery heat production of frog's muscle has been reinvestigated at  $0^\circ\text{C}$  under a variety of conditions.

The ratio of total energy to initial energy is consistently about 2, (a) in single contractions, followed to complete recovery, (b) in a long series of contractions during a steady state, where the accumulated recovery heat is measured in the interval between two stimuli. It is the same when maximal work is done as when the conditions are isometric.

During a steady state the rate of recovery heat production is nearly uniform (decreasing only slightly in the interval between two stimuli) and may be several times as great as the resting metabolic rate. The objection that the post-stimulation heat may be due merely to an increase in the resting metabolism is considered.

The allowance for heat lost in such experiments is discussed.

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**The reactions of the urinary bladder of the cat under conditions of constant pressure.** By J. MELLANBY, F.R.S., and C. L. G. PRATT. (*Received 12 May 1939*)

Provided that the anaesthesia is not too deep, the isotonic bladder of the cat shows continuous rhythmic contractions, the amplitude and frequency of which depend upon the hydrostatic pressure imposed upon the bladder. At any given pressure, the bladder volume remains constant. The greater the pressure, the larger the volume adopted by the bladder, but there is no simple relation between pressure and volume. Division of the nervi erigentes seriously interferes with the rhythm, whereas division of the hypogastric nerves either has no effect or else increases the amplitude and decreases the frequency of the contractions. Electrical stimulation of either set of nerves causes a diminution of volume, and that due to hypogastric stimulation is followed by a dilatation which is more marked at high than at low pressures. Adrenaline in small, moderate and large doses produces contraction, relaxation followed by contraction, and pure relaxation respectively. Acetyl choline causes contraction, followed, especially if the dose is large or the pressure is high, by relaxation. Atropine and ergotoxine produce a condition of immobility in the bladder. Experiments depending on the selective blocking of nerve impulses by these drugs are therefore of doubtful value.

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**Absorption of penetrating cosmic ray particles in gold.** By J. G. WILSON.  
(Communicated by P. M. S. Blackett, F.R.S.—Received 15 May 1939.)

Absorption measurements of the penetrating component of the cosmic radiation have been made by cloud chamber observations of the curvature change of tracks in a field of 10,000 G, using a gold absorber 2 cm. thick.

At low energies ( $E_s < 7 \times 10^8$  eV) the measurements indicate an absorption due entirely to ionization; from two tracks of very low energy ( $E_s < 2 \times 10^8$  eV) the increase of this absorption leads to an estimate of the mass of the mesotrons of  $\mu = (170 \pm 20) m_0$ . There is no indication of cross-sections for interaction of mesotrons with nuclear particles of comparable magnitude to that for absorption by ionization.

At higher energies ( $E_s > 7 \times 10^8$  eV) a few particles were observed which appeared to undergo greater absorption, and these are shown to be in agreement with our previous measurements. Experiments due to Ehrenfest make it unlikely that these particles belong to the predominant group of mesotrons, and it is probable that they are protons. Since the particle emerging from the gold plate is, in some cases, definitely not a proton, this hypothesis suggests a strong absorption of protons with the production of mesotrons in 2 cm. gold. A similar process has been suggested by Johnson for the production of mesotrons in the upper atmosphere.

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# ABSTRACTS

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4 JULY 1939

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**Calculated wave functions and energy values for X-ray terms of potassium.**  
By W. A. THATCHER. (*Communicated by D. R. Hartree, F.R.S.—Received 28 April 1939.*)

Self-consistent field calculations, without exchange, have been made for two states of the potassium atom ionized in an inner group, namely, for the configurations

$$K^{+2}[(1s)^1 (2s)^2 (2p)^6 (3s)^2 (3p)^4]$$

and

$$K^{+2}[(1s)^2 (2s)^2 (2p)^6 (3s)^2 (3p)^4].$$

The results are given in this paper. The perturbation of the wave functions of the outer electron groups on removal of an electron from an inner group is found to be considerable, so that, for example, the (3s) and (3p) wave functions of a K atom ionized in the (1s) group are more like those of a normal Ca atom than like those of a normal K atom. The results may be applied to take this perturbation into account in the theory of the Auger effect or of X-ray dispersion.

The application made in the present paper is to the calculation of X-ray ionization energies. The total energies of these configurations and of the normal state of  $K^+$  have been calculated by Slater's method, and the calculated values of  $\nu/R$  for the  $K$ ,  $L_{II}$  and  $L_{III}$  edges and  $K\nu$  doublet obtained and compared with the observed values. The agreement is very good for the  $K$  edge and good for the  $K$  lines but less so for the  $L$  edges.

A new method of evaluating one of the quantities,  $\gamma_k(nl, n'l'/r)$ , required into the evaluation of the energy integrals is given.

**The friction of clean metals and the influence of adsorbed gases. The temperature coefficient of friction.** By F. P. BOWDEN and T. P. HUGHES. (*Communicated by C. H. Desch, F.R.S.—Received 2 May 1939.*)

A method is described for measuring the kinetic friction between metal surfaces which have been freed from the oxide and surface films that are normally present. The removal of the films has a profound effect, and the kinetic friction between the outgassed metals may be twenty times greater than that observed for the same metals cleaned in air.

The addition of a trace of oxygen to the clean metal causes an immediate reduction in the friction. Adsorbed hydrogen and nitrogen have little or no effect. Although the friction is reduced by a single film, it is clear that polymolecular layers are necessary before a substance can act as an effective boundary lubricant for moving surfaces.

The temperature coefficient of friction between clean metals was determined over a wide temperature range. Most of the metals investigated show a small but regular decrease in the kinetic friction as the temperature rises. If the temperature causes excessive softening of the metal the friction may rise to a high value.

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**The mechanism of sliding on ice and snow.** By F. P. BOWDEN and T. P. HUGHES. (*Communicated by C. H. Desch, F.R.S.—Received 2 May 1939.*)

Experimental studies of the friction on ice surfaces have shown that the low frictions observed at temperatures near the melting point were due to lubrication by a thin water film at the points of contact between the sliding surfaces. The coefficient of kinetic friction was found to be independent of the load, apparent area of contact, and speed of sliding over a certain range. When the temperature of the ice was decreased, the friction rose markedly as the water film became more difficult to form. Using ski of various materials, it was observed that the friction depended very largely on the thermal conductivity of the ski. This result suggested that frictional heating played a large part in melting a water film during sliding; it had been previously considered that pressure melting was alone responsible for the formation of this water film.

Experiments with miniature and real ski on snow surfaces showed that the same general laws were obeyed as on ice surfaces. The higher frictions obtained on snow were attributed to the extra work done in displacing and compressing the snow crystals.

**Spontaneous rhythmic impedance changes in the trout's egg.** By M. J. HUBBARD and LORD ROTHSCHILD. (*Communicated by J. Gray, F.R.S.—Received 12 May 1939.*)

Unfertilized and fertilized eggs of the rainbow trout show spontaneous periodic changes of impedance. Measurements were made with an A.C. bridge and electron oscillograph as detector.

At an input frequency of 3000 cycles the maximum change in impedance per egg cycle corresponds to an approx. 0.4 % impedance change in the equivalent parallel resistance and capacitance network in the standard arm of the bridge. The maximum capacitance and resistance changes in the latter are approx. 4.0 and 0.4 % respectively. The effect occurs only after about 7 hr. immersion in water. The frequency is about 1.5 min. Killing the egg abolishes the effect. An increase in temperature increases the frequency and vice versa.

It is suggested that the impedance cycle consists of two components: (1) the main periodic change whose frequency is about 1.5 min., and (2) an in-phase periodic change of much smaller amplitude with a frequency of one-quarter that of the former.

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**The cause of multiple discharges in a Geiger-Muller counter.** By C. H. COLLIE and F. MORGAN. (*Communicated by F. A. Lindemann, F.R.S.—Received 12 May 1939.*)

The formation of double kicks in a Geiger-Muller counter has been investigated experimentally. It is shown that they can be produced by the presence of small amounts of xylene vapour in the counter, and that the double kick is due to the release of an electron from the cathode when the xylene ions are discharged  $10^{-3}$  sec. after the first discharge.

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**Investigations of the mechanism of the transmission of plant viruses by insect vectors.** By H. H. STOREY. (*Communicated by F. T. Brooks, F.R.S.—Received 16 May 1939.*)

When puncturing through a membrane into a fluid, *Cicadulina mbila* ejects saliva only when its stylets are in motion, and not when they are at rest and fluid is being drawn into them. This saliva sets to a gel and is moulded internally by the stylets to form a sheath. No other material of insect origin has been observed to flow from the stylets.

The salivary glands of infective insects, when inoculated into the abdomens of non-infective ones, caused a few of these to become infective. Comparative experiments with other organs from the insect support the interpretation that the salivary glands may contain virus, either in small quantities or occasionally.

Attempts to demonstrate virus in a fluid upon which many infective insects had fed were almost always negative. Only when infective and non-infective insects fed simultaneously on a film of fluid held between two membranes did a few of the non-infective insects become infective, and these never caused infection more than once in a series of tests.

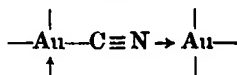
Results were similar with a maize leaf on which infective insects had fed. Simultaneous feeding on a small area of leaf alone caused a few non-infective insects to become infective, and these again were of low infective ability.

It is suggested that an infective *C. mbila* ejects virus in very small quantities, so that only rarely can another individual take up enough ejected virus to make it

infective and then only weakly so. The manner in which the virus is carried into the plant is still obscure; it is difficult to reconcile the view that the gelling saliva is the vehicle with the evidence of an earlier paper.

**The crystal structure of di-*n*-propylmonocyanogold.** By R. F. PHILLIPS and H. M. POWELL. (*Communicated by C. S. Gibson, F.R.S.—Received 19 May 1939.*)

Di-*n*-propylmonocyanogold crystallizes in the orthorhombic system,  $a_0 = 17.06$ ,  $b_0 = 22.36$ ,  $c_0 = 10.0$  Å, space group *Pca*. There are 16 molecules of the formula  $\text{AuPr}_2\text{CN}$  per cell. A Patterson analysis and Fourier syntheses on (001), (100) and (010), two of these non-centrosymmetric, have been made and show that the true molecule consists of four of these units. The molecule is based on a square with gold atoms at the corners, linked by CN groups along each side, and two propyl groups are attached to each gold atom. The length of the side is found to be 5.18 Å in accordance with the structure

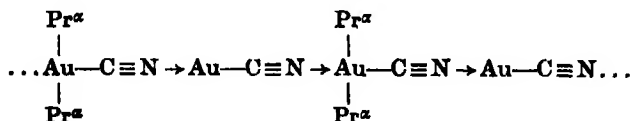


The four covalencies of the auric gold atom are at right angles to each other and coplanar, and the bond length is close to 1.3 Å. Although the syntheses are based on eye estimated intensities and the structure contains heavy atoms, the general positions of the largely overlapping propyl and cyano groups are clearly shown, especially in the centrosymmetric projection on (001). Special considerations are used to assign more definite sites to the lighter atoms in accordance with the projections, and the 144 atoms of the unit cell have been located. The bonds from the gold atoms to propyl groups all lie in the plane of the square, but the rest of the propyl groups are arranged out of this plane in a complicated way. Intermolecular distances are not less than 3.5 Å. The low symmetry of the structure is attributed to the difficulty of packing an awkward shaped molecule without leaving gaps.

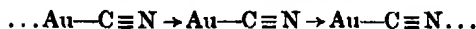
**Note on the constitution of cyano derivatives of gold.** By C. S. GIBSON, F.R.S. (*Received 19 May 1939.*)

The results of the investigation described in the previous paper make it possible to suggest structural formulae for the decomposition products of di-*n*-propylmonocyanogold which are more satisfactory than those originally put forward by the author.

It seems probable that the insoluble di-*n*-propyldicyanodigold produced by loss of half the *n*-propyl radicals from di-*n*-propylmonocyanogold has a chain structure represented thus:



By complete loss of the *n*-propyl radicals aurous cyanide is produced which in all probability has also a chain structure represented thus:



analogous to that of silver cyanide:

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**The action of ultra-violet radiation on barium and strontium azides.** By W. E. GARNER, F.R.S. and J. MAGGS. (*Received 22 May 1939.*)

Crystals of the alkaline earth azides are decomposed by ultra-violet light at room temperature. The threshold wave-length for this reaction is  $\sim 3000$  Å in agreement with the threshold for the absorption by the  $\text{N}_2$  ion in the solid state. There is an induction period before the evolution of nitrogen becomes measurable, after which the pressure increases at a linear rate.

Pretreatment of the crystals with ultra-violet produces changes in the length of the induction period and slopes of the  $\log p$ - $\log t$  time curves for the thermal reaction. For short times of illumination of the crystals,  $p = k_1 t^s$  and  $\tau = k_2/t_i^s$ , where  $\tau$  is the time of illumination and  $t_i$  the length of the induction period. This is interpreted as indicating that ultra-violet light produces centres from which nuclei grow, those centres requiring 3-4 barium atoms before a nucleus is formed. The centres are possibly *F* centres produced by the decomposition of  $\text{N}_2$  ions by the light. On prolonged illumination, nuclei are formed at room temperatures. Evidence of a solarization effect was obtained for high intensities of light.

The number of nuclei formed in the thermal treatment is enormously increased by previous exposure to ultra-violet light, and it is shown that ultra-violet light produces two types of centres from which the nuclei grow, one being confined to the action of wave-lengths less than 2360 Å.

The thermal decomposition of  $\text{BaN}_2$  is unaffected by fields of 1250 V/cm.

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**The thermal decomposition of crystals of barium azide.** By A. WISCHIN. (*Communicated by W. E. Garner, F.R.S.—Received 22 May 1939.*)

Measurements have been made of the rate of nuclear growth in the decomposition of crystals of barium azide and it has been found that the nuclei increase in radius at a linear rate. The activation energy for nuclear growth is 23.5 kcal., and the frequency of activation  $\sim 10^{14}$  molecular layers per second.

The numbers of nuclei increase as the third power of the time, which indicates that nuclei formation is a bimolecular process. The activation energy associated with this process is 74 kcal.

The pressure of the nitrogen evolved increases as the  $10^6$ - $10^8$  power of the time, which is in good agreement with that calculated from the rate of nuclear growth and the rate of increase in the numbers of the nuclei.

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**On the decomposition of metallic azides.** By N. F. MOTT, F.R.S. (*Received 22 May 1939.*)

A theoretical discussion of the decomposition of metallic azides, based on the Wagner-Schottky theory of ionic conductivity and the electron theory of solids. The main points discussed are: the formation of metallic nuclei and the nature of the induction period; the way in which the presence of nuclei catalyses the reaction; the action of ultra-violet light. The similarity between the decomposition of azides and the photochemical reduction of silver halides is pointed out.

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**The mechanical efficiency of frog's muscle.** By A. V. HILL, Sec.R.S. (*Received 24 May 1939.*)

A theoretical discussion is given of the mechanical efficiency (work/total energy) of frog's muscle. This is based on the heat of shortening, the heat of maintaining a contraction, and the constant  $b$  defining the effect of tension on the rate of energy liberation. Equations are given relating the efficiency to the speed of shortening, in a contraction at constant speed. The constants are known for frog's muscle at 0° C and absolute values of the efficiency are calculated.

Experiments are described using a "protected" thermopile and a constant-speed ergometer, from which it appears that the relation between efficiency and speed is of the predicted form, and that the maximum efficiency and the optimum speed are very close to the predicted values.

The observed maximum efficiency (40 %) of the initial process corresponds to an efficiency of 20 % for the whole muscular cycle including recovery. This is not much less than the maximum efficiency in man. The efficiency of human muscular movements is considered.

A curious equality (or approximate equality) is discussed; the rate of heat production in maintaining a contraction at constant length is about equal to the extra heat of shortening at velocity  $b$ . This may have some significance not yet apparent.

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**The electrical conductivity of thin films of mercury.** By E. T. S. APPLE-YARD and J. R. BRISTOW. (*Communicated by A. M. Tyndall, F.R.S.—Received 25 May 1939.*)

The electrical resistance of mercury films deposited in very high vacua on surfaces cooled to various temperatures between 20·4 and 90° K has been investigated. The thickness of the films has been estimated by direct weighing to 3 %. The results indicate that the delayed appearance of conductivity as deposition proceeds is due to agglomeration of the mercury into solid droplets, and that the cause of the sudden onset of conductivity is the touching of these droplets. The temperature coefficients of resistance of these films are all positive and reversible after annealing at the highest point in the temperature range. From a study of these coefficients it



is concluded that films above 500 Å in thickness deposited at 64° K or lower can be regarded as coherent slices of metal, differing only from the bulk metal in being strained and in possessing a high residual resistance which can be modified by annealing treatment.

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**Superconductivity of thin films. I. Mercury.** By E. T. S. APPLEYARD, J. R. BRISTOW, H. LONDON and A. D. MISENER. (*Communicated by A. M. Tyndall, F.R.S.—Received 25 May 1939.*)

Mercury films prepared in high vacua have been examined in the superconducting state. The transition temperature from the normal to the superconducting state in films previously annealed at 90° K or more is very close to that of the bulk metal. On the other hand, films deposited at 4.2° K and not afterwards annealed show a displacement of the transition temperature downwards by about 0.2° K. In neither case is there evidence of any variation of transition temperature with the thickness of the films.

The critical longitudinal magnetic fields required to restore normal conductivity in the films at various temperatures have been determined.

If  $H_f$  is the critical magnetic field for a film at a definite temperature and  $H_M$  that for the bulk metal at the same temperature, it is found that at all temperatures  $H_f/H_M$  is larger than unity, and increases rapidly as the film diminishes in thickness. For a film of a definite thickness  $H_f/H_M$  increases steeply as the transition temperature is approached. It is inferred from this result that the depth of field penetration likewise increases steeply as the transition temperature is approached.

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**The spectrum of rubidium hydride, RbH. I. Analysis.** By A. G. GAYDON and R. W. B. PEARSE. (*Communicated by A. Fowler, F.R.S.—Received 26 May 1939.*)

A band spectrum attributed to rubidium hydride, RbH, has been observed in a discharge tube source containing metallic rubidium and hydrogen. The spectrum has been photographed on a 20 ft. concave grating spectrograph, and a rotational and vibrational analysis has been made.

The spectrum is of the "many-line" type characteristic of the alkali-metal hydrides, and is produced by a  $1^1\Sigma \rightarrow 1^1\Sigma'$  electronic transition.

The band system is strongly degraded to longer wave-lengths, corresponding to a large change of vibrational frequency;  $\omega_e$  is 936.77 cm.<sup>-1</sup> for the ground electronic state and 244.6 cm.<sup>-1</sup> for the excited state.

The constants associated with the vibration and rotation of the molecule have been calculated for the ground and excited electronic states. The excited state shows anomalies similar to those observed for the other alkali-metal hydrides.

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**The spectrum of rubidium hydride, RhH. II. Potential curves, wave functions and intensity distribution.** By A. G. GAYDON and R. W. B. PEARSE. (*Communicated by A. Fowler, F.R.S.—Received 26 May 1939.*)

The intensity distribution among the various vibrational transitions of the electronic emission spectrum of rubidium hydride is described; in addition to the normal Franck-Condon parabola, which in this case is very open, there are very well-marked secondary and tertiary parabolae.

To account for these subsidiary parabolae it is necessary to apply the methods of wave mechanics. As rubidium hydride is a particularly favourable example, an attempt has been made to compare the observed intensity distribution with that expected theoretically.

The potential functions have been calculated from the molecular constants derived from the analysis of the spectrum, and the potential curves are drawn and discussed briefly.

The wave functions for the various vibrational levels of a harmonic oscillator have been calculated for the two electronic states, and these have been distorted, by a simple method, to correspond approximately to the potential curves.

Using these wave functions, the intensity distribution has been calculated. It is in good general agreement with the observations, but shows some discrepancies in detail. The various assumptions and approximations made in making the calculations are examined. It is concluded that such discrepancies as exist are probably to be attributed to the approximate method of distorting the wave functions of the harmonic oscillator to correspond to the potential curves.

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**Morphogenesis and metabolism: studies with the cartesian diver ultra-micro-manometer. V. Aerobic glycolysis measurements on the regions of the amphibian gastrula.** By J. NEEDHAM, V. ROGERS and S. C. SHEN. (*Communicated by Sir Frederick Hopkins, F.R.S.—Received 30 May 1939.*)

The cartesian diver manometer has been applied to the measurement of aerobic glycolysis, following the two-cup method of Warburg. After a description of the calibration and testing of the method, which is applicable to total gas changes of the order of one-tenth of one cubic millimetre, it is shown that the aerobic glycolytic rate of all the regions of the amphibian gastrula is extremely low, approaching zero, and hence that the Pasteur reaction is equally efficient in the organisation centre and the ventral ectoderm. The absence of any difference in respiratory rate between these two regions, previously observed in gastrulae of toad and axolotl, is here confirmed by a different method for the frog. Similarly, the difference in respiratory quotient between the two regions previously observed in axolotl gastrulae, is here confirmed by a different method for the frog. It seems legitimate, therefore, to regard the general picture of metabolic changes in gastrulae found in the present series of papers as probably valid for most Amphibia.

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**On relativistic wave equations for particles of arbitrary spin in an electromagnetic field.** By M. FIERZ and W. PAULI. (*Communicated by P. A. M. Dirac, F.R.S.—Received 31 May 1939.*)

The force-free theory of particles with arbitrary spin values already published by one of the authors is generalized to the relativistic wave equations of such particles in an electromagnetic field, with a preliminary restriction to the c-number theory. The spin values  $3/2$  and  $2$  are treated in detail, and for the general case it is merely proved that consistent wave equations exist. The consistency of the system of field equations is attained by deriving them from a Lagrange function containing suitable additional terms which depend on new auxiliary quantities. All the differential equations of the field are derived by variation of the action integral, and the vanishing of the auxiliary quantities in the absence of an external field is made to follow as a consequence of them.

In the special case of zero rest-mass there exist identities between the equations, which are now invariant under a group of transformations which is the generalization of the group of gauge transformations in Maxwell's theory. In the particular case of spin  $2$ , rest-mass zero, the equations agree in the force-free case with Einstein's equations for gravitational waves in general relativity in first approximation; the corresponding group of transformations arises from the infinitesimal co-ordinate transformations.

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**The reflexion of very long wireless waves from the ionosphere.** By M. V. WILKES. (*Communicated by E. V. Appleton, F.R.S.—Received 1 June 1939.*)

This paper is concerned with the theoretical interpretation of the experimental data already published by Best, Ratcliffe and Wilkes and by Budden, Ratcliffe and Wilkes on the reflected wave received via the ionosphere at short distances (about 90 km.) from the British Post Office transmitter at Rugby (wave-length 18,800 m.; 16 kcy./sec.). It is shown that the diurnal changes in reflexion height are in agreement with what would be expected if reflexion took place from the bottom of a region of the type described by Chapman, in which the ionization is in quasi-equilibrium with the changes in zenith angle of the sun. The polarization and amplitude of the wave reflected from a layer of ionized gas in a magnetic field when a plane polarized wave of wave-length comparable with the dimensions of the layer is incident on it are discussed, and in particular the type of wave reflected from a Chapman region is deduced. It is shown that the observed constancy of the amplitude of the downcoming wave until near the end of the sunset period leads to the conclusion that in the reflecting region the frequency of electronic collisions is not greater than about  $2 \times 10^6$  per sec., and the possibility of collisional absorption taking place below the reflecting level is discussed. The question of whether or not the "Lorentz term" is to be included in the magneto-ionic equations is considered, and it is shown that the long-wave evidence, though not conclusive, appears to be against including it.

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**Studies on the insect virus relationships of Hy. 3 virus, potato virus Y and cucumber virus 1 when transmitted by *Myzus persicae* (Sulz.), *Myzus circumflexus* (Buckt.) and *Macrosiphum gei* (Koch). By F. M. ROBERTS and M. A. WATSON. (Communicated by Sir John Russell, F.R.S.—Received 1 June 1939.)**

Three strains of *Hyoscyamus* virus 3, two of cucumber virus 1, and potato virus Y, were tested for their transmissibility by the aphides, *Myzus persicae*, *M. circumflexus* and *Macrosiphum gei*. The efficiency of the vectors in transmitting all the viruses increased with increasing time of fasting before feeding on the infected plants. Their efficiency decreased as the time of feeding on the infected plants increased. The most probable explanation of these effects is that the viruses are inactivated by some substance produced by the aphides when feeding.

The most successful vector on the whole was *Myzus persicae*, and the least successful was *Macrosiphum gei*, but the relative efficiency of the vectors varied with the different viruses, indicating that their degree of success depended upon several interacting factors. The most important of these factors appeared to be:

- (a) The concentration of virus in the host plant.
- (b) The localization of virus in the host plant.
- (c) The capacity of the vector for inactivating the virus.

The viruses which have thus been shown to be similar in their insect-virus relationships are also similar in their physical properties, and there are many other aphid-transmitted viruses which resemble them in this respect. It is suggested that such viruses may form a natural group, with the same type of vector-virus relationship. This relationship appears to be a complex one, and it is unlikely that the viruses are mechanically transmitted.

**The number of configurations of an assembly with long-distance order. By T. S. CHANG. (Communicated by R. H. Fowler, F.R.S.—Received 1 June 1939.)**

Starting from the equilibrium properties of an ideal alloy with a superlattice of the type *AB* and with arbitrary compositions which are obtained by application of Bethe's method, we have found the partition function of the alloy by a process which is simply the reverse of deducing the equilibrium properties from the partition function. It is shown that if this partition function is expanded in powers of  $(kT)^{-1}$ , it agrees with the corresponding rigorous expression up to the power  $(kT)^{-1}$ .

From this partition function, we find an approximate expression for the number of ways of arranging  $\frac{1}{2}N(\theta + \theta')$  particles upon a lattice of the type *AB* consisting of *N* sites in total, with  $\frac{1}{2}N\theta$  particles on one sublattice and  $\frac{1}{2}N\theta'$  particles on the other, producing *X* pairs of nearest neighbours formed by the particles. This is given by an expression involving  $d = 1 - \theta - \theta'$  and  $q = 4X/zN$ , *z* being the number of nearest neighbours of a site. The exact value of this number is evaluated for the special case in which the *N* sites are on a straight line, and is found to agree with the approximate formula exactly.

**The scattering of alpha particles in helium.** By S. DEVONS. (*Communicated by W. L. Bragg, F.R.S.—Received 1 June 1939.*)

The scattering of alpha particles in helium has been investigated for scattering angles of  $27^\circ$  and  $38.5^\circ$ , and for energies up to 8.5 MeV. It is found that the results are consistent with the existence of a broad level of  $\text{Be}^4$  with spin two quantum units, and with an energy of about 3.3 MeV in excess of the ground state, which is assumed to possess a mass equal to that of two alpha particles. The results for alpha particle energies greater than 7.0 MeV are not very accurate owing to the low intensity of the sources of thorium active deposit available.

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**A contribution to the theory of diffusion in non-ideal liquids and membranes.** By J. F. DANIELLI. (*Communicated by Sir Frederick Hopkins, F.R.S.—Received 5 June 1939.*)

This paper has been written to cope with certain physical problems encountered in the study of the permeability of living cell membranes to penetrating molecules.

No theory of diffusion through liquids and solids has previously been presented which takes account of the structure of the diffusion medium. In this paper the point of view is adopted that all such media can be represented to a first approximation by an appropriate system of potential energy barriers, to cross any one of which a molecule must have more than a minimum kinetic energy—the activation energy of diffusion.

In § 1 certain cases are discussed where, within limits, the activation energy of diffusion may be ignored (actually, assumed identical for all molecules).

In § 2 it is assumed that the rate of diffusion over a single potential energy barrier is determined by a simple exponential equation of the form

$$\text{rate} = r\phi (kT/2\pi m)^{1/2} \exp(-mv_0^2/2kT) C,$$

where  $r$  is a constant,  $\phi$  is the probability that a molecule having at least the kinetic energy  $\frac{1}{2}mv_0^2$  will actually diffuse,  $\frac{1}{2}mv_0^2$  is the activation energy of diffusion over the potential energy barrier,  $m$  is the mass of the diffusing molecule,  $k$  is the gas constant per molecule,  $T$  is the absolute temperature,  $C$  is the concentration of the diffusing molecules. Various interfaces are taken as representing single potential energy barriers, and it is shown that to a first approximation this exponential law accounts for the temperature variation of the rate of diffusion across such interfaces, provided no changes of structure, such as solidification, are involved.

In § 3 the same equation is applied to the diffusion of ions and molecules in water. It is shown that, to a first approximation, the equation accounts for the variations in rate of diffusion found with different ions and molecules.

In § 4 this approximate equation is applied to the problem of diffusion through a thin fatty membrane,  $10^{-6}$  cm. thick (the cell membrane). An expression is obtained for the permeability of this membrane, and it is shown that for a molecule such as glycerol the main resistance to diffusion lies at the oil-water interface, and that the resistance in the interior of the fatty layer may, by comparison, be neglected.

In § 5 is discussed some of the assumptions made in the previous sections.

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**Wave form, energy and reflexion by the ionospheres of atmospherics.**

By T. H. LABY, F.R.S., J. J. McNEILL, F. G. NICHOLLS and A. F. B. NICKSON. (*Received 6 June 1939.*)

The wave form of the electromagnetic pulse radiated from a lightning flash, known as an atmospheric, has been studied.

The atmospherics are received on a vertical aerial which is connected through an aperiodic amplifier to a cathode ray tube. The spot on the screen of the tube is photographed on a film fixed to the external surface of a cylindrical drum which rotates at a uniform peripheral speed.

Many hundreds of wave forms have been recorded from atmospheric sources 70 to 1500 km. distant, and incidental to the observations we have made, evidence has been obtained of the reflexion of atmospherics at an ionized layer. Such records show the intervals of time elapsing between the arrival of the first pulse along the ground, and the various reflected pulses.

From the simple theory and methods of reduction given in the paper, it is possible to determine the height of the reflecting ionized layer, and the distance of the flash. When this is done, the height of the layer is found to be between 53 and 82 km., values in reasonable agreement with the lower limits of the *E* layer.

The observations are consistent with the sky wave and the ground wave, having the same velocity to 0.7 %.

Oscillograms of typical atmospheric wave forms are shown, together with a possible interpretation of many of them. The assumption is made that the electrical discharge which radiates an atmospheric is a damped oscillation with a period determined by the instantaneous resistance, inductance and capacity.

The relation between the distance of an atmospheric source and its field strength is found to be linear and figures are given for the peak power and the total energy radiated as found from representative examples.

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**On fluctuations in electromagnetic radiation. By M. BORN, F.R.S. and K. FUCHS. (*Received 6 June 1939.*)**

A mistake (pointed out by Dr Fierz) in the former paper, *Proceedings A*, 170, 252, is corrected.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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19 JULY 1939

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**Defect lattices in some ternary alloys.** By H. LIPSON and A. TAYLOR.  
(Communicated by A. J. Bradley, F.R.S.—Received 9 June 1939.)

In the Ni-Al system, the solution of Al in the  $\beta$ -Ni-Al (body-centred cubic) structure has been shown to be accompanied by an omission of Ni atoms from some of the lattice points. A study of the Cu-Ni-Al system shows that the omission of atoms begins at a line of constant ratio of valency electrons to atoms, and this is supported by evidence from the systems Fe-Cu-Al and Fe-Ni-Al.

These results may be explained by the accommodation of electrons in the Brillouin zone. If the electrons fill the zone to a given energy level (which, in this case, is very near to the inscribed sphere of the zone), then the number of electrons per unit cell should be constant. It is shown that this is so for the Ni-Al system, but it is not true for the Cu-Ni-Al system, the number of electrons in the zone increasing as Cu is added. It is presumed that the energy relationships are affected by the atomic size factor, Cu being a larger atom than Ni.

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**The magnetic susceptibility of nickel chloride.** By H. R. NETTLETON and S. SUGDEN, F.R.S. (Received 10 June 1939.)

A solution of nickel chloride appears to be very suitable as a standard for magnetic measurements since the nickel content can be accurately determined by standard analytical methods and the molar susceptibility is independent of dilution. A new determination of the molar susceptibility has been made using null methods to determine the field in the gap of a specially designed magnet followed by measurements of the susceptibility of carefully analysed nickel chloride solutions by the Gouy method.

The mean value found  $10^4 \chi_{30^\circ} = 4436$  is in good agreement with that of earlier workers, viz. 4448 (Weiss and Bruins 1915), 4423 (Brant 1921). These earlier determinations were made by different physical methods so that the susceptibility of nickel chloride is now known with considerable accuracy.

A brief discussion is appended on the use of solutions of nickel chloride to calibrate vessels for measuring magnetic susceptibilities.

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**Shower production by penetrating cosmic rays.** By A. C. B. LOVELL.  
(Communicated by P. M. S. Blackett, F.R.S.—Received 12 June 1939.)

A counter-controlled cloud chamber has been used to investigate cosmic ray showers under thick layers of lead. 5800 photographs, containing 463 electronic showers of not less than five particles, have been taken in a magnetic field of 750 gauss.

An analysis of the evidence and probabilities for various possible processes of shower production is carried out, and it is shown that the showers observed can be accounted for as cascade showers, produced by an electron which has been knocked on in a direct collision with a mesotron. It is unnecessary to assume the occurrence of other processes of appreciable cross-section.

Due to the influence of the incident energy spectrum of mesotrons, the majority of large showers are produced as fluctuations, and in general, the lowest mesotron energy which is able to produce a shower of  $N$  particles is only slightly lower than the most probable energy for the production of such a shower.

A search has been made to find showers, or single secondary particles, produced by neutral particles in cosmic radiation, with negative results.

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**The lamellar structure of potash-soda feldspars.** By S. H. CHAO and W. H. TAYLOR. (Communicated by W. L. Bragg, F.R.S.—Received 13 June 1939.)

A previous general survey of orthoclase-micropertthites suggested the existence of different types of lamellar structure according as the proportion of soda feldspar is less than or greater than 30 % approximately. A new and more detailed examination confirms this, and suggests that the low-soda structure comprises monoclinic potash feldspar with triclinic soda feldspar lamellae in the mutual orientation characteristic of pericline twins, while the high-soda structure comprises monoclinic potash feldspar with triclinic soda feldspar lamellae oriented in accordance with the albite twin-law. The constitution of the soda feldspar and the bearing of the new data on the general theory of micropertthitic structures are discussed.

An examination of a Korean moonstone, apparently identical with material in which Ito claims to have established the existence of *monoclinic* soda feldspar, reveals a lamellar structure of the low-soda type described above. Ito's results are discussed.

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**Self-consistent field with exchange for carbon.** By A. JUCYS. (*Communicated by D. R. Hartree, F.R.S.—Received 14 June 1939.*)

Solutions of Fock's equations for the self-consistent field with exchange have been carried out for the normal states of  $C^{+4}$  and  $C^{+2}$ , and for all three configurations ( $^3P$ ,  $^1D$  and  $^1S$ ) arising from the normal configuration of neutral C.

For neutral C, the energies of the  $(2s)(2p)$  have been calculated both without and with the inclusion of the term arising from the superposition of the  $(2p)^4$  configuration on the normal  $(2s)^3(2p)^2$  configuration.

Tables of wave functions and energies are given.

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**On the intensities of electron diffraction rings.** By M. BLACKMAN. (*Communicated by G. P. Thomson, F.R.S.—Received 14 June 1939.*)

The reflexion of electrons by a thin film is examined from the point of view of the dynamical theory in the Laue case. The formulae are used to obtain a criterion which determines when the scattering is sufficiently small to allow the kinematical theory to be applied; it is found that this theory is not in general applicable to the thin films used in work with fast electrons. The total intensity scattered from a film when it is turned through its range of reflexion is also found and the result is used to obtain an approximate intensity function showing how the intensities of the diffraction rings from a polycrystalline film will vary when the scattering is sufficiently large for the kinematical theory to be inapplicable. This intensity function is tested by comparing it with the experimental intensity function found for copper and silver. It is found possible to obtain a good fit with very reasonable assumptions as to the average crystal size.

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**A mathematical analysis of the distribution in maize of *Heliothis armigera* (Huebner) [= *obsoleta* F.].** By MARJORY G. WALKER, PH.D. (*Communicated by W. R. Thompson, F.R.S.—Received 22 June 1939.*)

The distribution among maize plants of the eggs of the American boll-worm, *Heliothis armigera* (Huebner), is discussed and analysed.

The problem is considered in relation to what is known of the connexion between the state of development of maize plants and their attractiveness to ovipositing boll-worm moths. The actual frequency distribution of the eggs suggests a random, as opposed to a uniform, distribution, but it is shown that the conditions required for a pure mathematical random distribution cannot be satisfied. Because the maize plants differ from one another in absolute degree of attractiveness at any one time, and in relative degree of attractiveness with the passing of time, it is not true that every plant has the same chance of receiving any particular egg.

It is demonstrated that a mathematical theory, which is essentially one of random distribution, but which incorporates a modification to allow for the varying degrees of attractiveness of the plants, gives a fairly good representation of the egg distribution found in the field.

It is pointed out that the method of mathematical analysis employed is only a rough approximation to what it is really intended to express, and that it cannot deal adequately with the essential difficulty of the problem—the fact of the continuous change in nature.

For the further study of problems of animal distribution it is desirable that the mathematical theories should be improved.

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**A note on the mechanism of boundary lubrication suggested by the static friction of esters.** By A. FOGG. (*Communicated by C. G. Darwin, F.R.S.—Received 22 June 1939.*)

The note gives the results of static friction measurements of steel surfaces lubricated with series of methyl and ethyl esters. The series of ethyl esters was chosen to obtain a relation between coefficient of friction and molecular weight, and an unexpected feature of the relation obtained is a maximum value of coefficient of friction at a molecular weight of about 120.

A suggested explanation of this phenomenon is given by making certain assumptions regarding the structure and method of attachment of the molecules to the surface.

On the basis of these assumptions, the behaviour of a series of methyl esters was predicted before making tests and the results of the tests subsequently carried out agree with the forecast.

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**The microscopic analysis of intermediate phases in some age-hardening alloys.** By M. L. V. GAYLER. (*Communicated by C. H. Desch, F.R.S.—Received 22 June 1939.*)

A microscopic analysis has been carried out at high ageing temperatures on high-purity aluminium alloys containing 4% copper and 4% copper + 0.5% magnesium and also on an alloy of the duralumin type, but without manganese, containing 4% copper, 0.5% magnesium, 0.3% silicon and 0.1% iron.

In the 4% copper alloy, plates of the intermediate phase,  $\alpha$ -CuAl<sub>2</sub>, have been observed on (100) planes partly or completely changed to  $\beta$ -CuAl<sub>2</sub>. It is deduced, therefore, that  $\alpha$ -CuAl<sub>2</sub> is a polymorphic form of  $\beta$ -CuAl<sub>2</sub> and is metastable at temperatures of ageing below the quenching temperature.

A second intermediate phase has been identified, during ageing, together with  $\alpha$ -CuAl<sub>2</sub> on (100) planes in the alloy containing 4% copper and 0.5% magnesium. This new intermediate phase also differs from another phase observed when the alloy of the duralumin type is aged.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

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**The particle aspect of meson theory.** By N. KEMMER. (*Communicated by S. Chapman, F.R.S. —Received 22 June 1939.*)

It is shown that a reformulation of the meson equations is helpful in the interpretation of the meson as a localized particle. Instead of using the usual tensor form, the wave equations are stated as

$$\frac{\partial}{\partial x_\mu} \beta_\mu \psi + \frac{mc}{\hbar} \psi = 0,$$

where the  $\beta_\mu$  are operators completely defined by a set of commutation rules first given by Duffin (1938). The theory can be developed in strikingly close correspondence to Dirac's electron theory, practically all the definitions of which find their exact counterpart, e.g. spin, magnetic moment, etc. The algebraic properties of the  $\beta_\mu$  are studied in detail, a comparison with other similar formulations is given and the limiting non-relativistic theory is developed. The formalism proves simple to handle and is expected to be useful in all calculations primarily concerned with the particle aspect of the meson.

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**Stress systems in aeolotropic plates. I.** By A. E. GREEN, and G. I. TAYLOR, F.R.S. (*Received 22 June 1939.*)

Equations which can be used for a system of generalized plane stress in a plate whose material has any kind of aeolotropy have been obtained recently by Huber. When the material has two directions of symmetry at right angles in the plane of the plate the equation for the stress function takes a comparatively simple form. In the present paper solutions in polar co-ordinates of this equation are obtained which give

single-valued expressions for the displacements, and these solutions are applied to the problem of an isolated force acting at an internal point of an infinite aeolotropic plate. The stress distributions due to such a force acting at a point of certain highly aeolotropic materials such as oak and spruce are represented by polar diagrams.

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**Stress systems in aeolotropic plates. II.** By A. E. GREEN. (*Communicated by G. I. Taylor, F.R.S.—Received 22 June 1939.*)

In a previous paper by G. I. Taylor and the present writer, generalized plane stress systems were examined for infinite aeolotropic plates. In the present paper formulae are obtained for generalized plane stress systems in an infinite aeolotropic strip and also in a semi-infinite plate. In particular, a solution is given for the general problem of any force acting at any point either within or on the boundary of such plates. The stresses due to any distribution of force may be deduced by integration.

When a force acts on the boundary of a semi-infinite plate the stresses may be deduced from our general results, but this case is dealt with independently by solving the problem of an isolated force at the vertex of a wedge. The method used here differs from that used by Michell who was the first to solve this problem.

The stress distributions due to forces either within or on the boundary of a semi-infinite plate are illustrated numerically for a specimen of spruce wood which is highly aeolotropic.

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**The equilibrium and stability of thin aeolotropic plates.** By A. E. GREEN. (*Communicated by G. I. Taylor, F.R.S.—Received 22 June 1939.*)

An equation is established governing the equilibrium of any thin aeolotropic plane plate which is bent by transverse forces and also an equation for the stability of any thin aeolotropic plane plate under forces in its plane. The results are obtained by an elementary approximate method which is equivalent to that used by Huber who was the first to give the equation for the equilibrium of any thin aeolotropic plane plate which is bent by transverse forces. Some of the more elementary problems of equilibrium and stability are solved. Numerical results are given for the stability of thin rectangular sheets of oak and spruce woods when they are acted on by edge thrusts, and also for the stability of a rectangular sheet of spruce under shearing forces.

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**Behaviour of the crystalline structure of brass under slow and rapid cyclic stresses.** By P. L. THORPE, *Engineering Department*, and W. A. WOOD, *Physics Department, National Physical Laboratory, Teddington, Middlesex.* (*Communicated by G. W. C. Kaye, F.R.S.—Received 1 July 1939.*)

The stress-extension relationships for an annealed brass have been measured for static tensile stress and for a symmetrical alternating tension-compression applied at a frequency of 2200 cyc./min., and the mechanical properties indicated by the

curves have been studied in relation to the changes in crystalline structure of the grains as shown by the X-ray diffraction method. It is shown that the dispersal of the grains into widely oriented crystallites, which constitutes the physical characteristic of the change at the yield point under ordinary static or slow cyclic stress, is entirely suppressed under the same regions of cyclic stress when applied at the high frequency, and that, after the cyclic stressing has ceased, this suppression persists up to a static load equal to the maximum of the prior stress cycle employed; the mechanical measurements indicate a corresponding inhibition of the primitive yield point. A further result is obtained by a precise comparison of the dimensions of the atomic lattice of specimens subjected to cycles of an unsafe range of stress; these show that an appreciable internal strain of the nature of a volume expansion is built up in the lattice during the rapid cyclic stressing, and it is considered that it is this distortion which is associated with the inhibition of the dispersed crystallite formation and rise in primitive static yield point.

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**A quantitative study of pleochroic haloes. IV. New types of haloes.** By G. H. HENDERSON and F. W. SPARKS. (*Communicated by J. Chadwick, F.R.S.—Received 3 July 1939.*)

Four types of haloes, provisionally designated as *A*, *B*, *C* and *D* are described, three of them for the first time. These haloes have one, two, three and one rings respectively. Measurements of ring radii are given. The mode of occurrence of the haloes is described. It is shown that they are probably due to  $\alpha$  particles from certain later members of the uranium family, whose lives are exceedingly short on a geological time scale.

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**A quantitative study of pleochroic haloes. V. The genesis of haloes.** By G. H. HENDERSON. (*Communicated by J. Chadwick, F.R.S.—Received 3 July 1939.*)

Six types of pleochroic haloes are discussed in this and the preceding paper, divided into two classes "active" and "extinct". To the former class belong uranium and thorium haloes; to the latter class types designated tentatively *A*, *B*, *C* and *D*.

To account for extinct types of haloes a hypothesis is advanced that they originate from hydrothermal solutions. Such solutions diffused through conduits or clefts in the biotite crystal and particular radioactive elements of the uranium family were deposited from them at certain centres of precipitation. By a continuous process there was thus incorporated in a halo nucleus a quantity of a short-lived element sufficient to produce a halo. In this way all four extinct types can be explained. In active haloes this method of halo formation also occurred but the possibility is also open that the halo nucleus was formed prior to the crystallization of the biotite. All halo rings can be accounted for by known types of  $\alpha$  particles and no evidence is found for unknown elements which may once have existed in the earth and have now disappeared.

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**The actions of iodine and hypiodous acid on pepsin.** By J. ST L. PHILPOT and P. A. SMALL. (*Communicated by R. A. Peters, F.R.S.—Received 3 July 1939.*)

When iodine acts on pepsin the initial ratio of fall in Folin blue value to fall in peptic activity decreases with increasing pH and temperature, reaching a very low figure at pH 6 and 38° C. The same occurs with "diazopepsin", in which the reactive tyrosine groups have previously been attacked by nitrous acid.

When pepsin is treated with small amounts of hypiodous acid the initial ratio of fall in Folin blue value to amount of hypiodous acid is much lower than for tyrosine, being practically nil at pH 5.4. The hypiodous acid required to produce a just perceptible fall in Folin blue value is sufficient to destroy most of the peptic activity.

It is concluded that some groups other than tyrosine are concerned and reasons are given for excluding other amino-acids.

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**Thermal ionization of barium.** By B. N. SRIVASTAVA, M.Sc. (*Communicated by M. N. Saha, F.R.S. —Received 3 July 1939.*)

In this paper a new method has been described for experimentally studying the thermal ionization of gases. The apparatus used has already been described in detail by Saha and Tandon elsewhere. Thermal ionization of barium vapour has been investigated at various temperatures and the equilibrium constant and the effective energy of ionization have been calculated. The results obtained agree, within the limits of experimental error, with the theory of thermal ionization. The results also support the usual interpretation of the well-known barium anomaly in the solar spectrum, and yield for the effective ionization energy at 1500° C a value of 114.1 kcal. though the ionization potential of barium is 5.19 V corresponding to 119.5 kcal. The theoretical value for this quantity at 1500° C is 114.4 kcal. The agreement is therefore quite satisfactory.

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**The influence of diabetogenic anterior pituitary extracts on the islets of Langerhans in dogs.** By K. C. RICHARDSON. (*Communicated by Sir Henry Dale, F.R.S.—Received 4 July 1939.*)

The experimental investigation of the aetiology of diabetes mellitus in intact animals has been made possible by the discovery that the daily injection into dogs, of large amounts of a crude anterior pituitary extract results in a clearly defined diabetic condition which may continue after the injections cease and become permanently established.

The histological condition of the pancreas, in a series of dogs so treated, has been examined to determine the influence of the diabetogenic anterior pituitary extract, particularly on the beta cells of the islets of Langerhans. Partial or complete degranulation of the majority of the beta cells, accompanied by hydropic degeneration in

individual beta cells, was found in the material taken during the period of injections with pituitary extract, when the dogs were exhibiting temporary symptoms of diabetes. A characteristic vacuolation of the intralobular duct epithelium was also observed.

Vacuolation of the duct epithelium and slight loss of cytoplasmic granules in beta cells, occurred in the pancreas of dogs treated with fractionated anterior pituitary extracts which failed to produce diabetic symptoms.

During the prolonged period of permanent diabetes, the islets of Langerhans may remain intact and show little change beyond loss of cytoplasmic granules in the beta cells, or they may undergo atrophy by a reduction to small groups of alpha cells, or by complete or partial replacement by hyaline material.

In dogs which become refractory to increased dosages of anterior pituitary extract and fail to exhibit permanent diabetic symptoms, there was evidence of hyperplasia in the islets of Langerhans.

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#### X-ray crystallography and the chemistry of the steroids. I. By J. D. BERNAL, F.R.S., D. CROWFOOT and I. FANKUCHEN. (*Received 5 July 1939.*)

A survey has been made of the X-ray crystallography of some eighty sterol derivatives belonging mainly to the cholesterol and ergosterol series but including also calciferol and other photo-derivatives of ergosterol and some higher plant and animal sterols. The measurements are recorded in four tables and include determinations of unit cell size, space group and some data on the crystal morphology and optics. In three cases,olesteryl chloride, bromide andolesteryl chloride hydrochloride, Patterson projections have also been derived from the intensities of the X-ray reflexions of the hol planes. These confirm earlier deductions on the shape and size of the sterol molecules, proving that these are roughly lath-shaped,  $20 \times 7 \times 4$  Å, and the details of the patterns can also to some degree be correlated with the actual arrangement of the carbon atoms in the sterol ring system and with the positions of the chlorine and bromine atoms. The arrangement of the molecules in the crystal units is closely that given by the preliminary examination from the optic orientations and this has therefore been employed to suggest in each of the remaining sterol crystal structures the probable molecular arrangement.

Among the eighty compounds studied a number of different types of crystal structure appear. An attempt is made to group these in a general geometric classification depending upon the relative orientation of the molecular axes, thickness, width, and length to the crystallographic axes  $a$ ,  $b$  and  $c$ . Altogether 104 different sterol structures are included in the classification and of these 77 fall into the second of the three main groups described above, which includesolesteryl chloride and bromide and has been called the normal type of sterol structure.

The crystallographic measurements as a whole are discussed in their bearing on specific chemical problems under the following headings:

(a) *Characterization and identification.* The X-ray data have been applied particularly among the higher plant and animal sterols both to identify individual

constituents (e.g. stigmasterol in phytosterol mixtures), to distinguish additional crystal forms (e.g. two types of cerovisterol) and to characterize new sterols and relate these to previously known compounds (e.g. a sterol from rubber).

(b) *Molecular weight determinations.* Molecular weight measurements have been carried out on coprosterone, ergotetraene,  $\gamma$ -spinasterol acetate, ergosterol  $H_2O$ , stigmasterol  $H_2O$ ,  $\gamma$ -sitosterol  $H_2O$  and a sterol from rubber. In the case of crystals of cholestanol, ergostadiene triol and 14-cholestene 7-ol, the measurements are used to estimate water of crystallization.

(c) *The stereochemistry of the carbon skeleton.* The X-ray measurements indicate that the general configuration of the sterol ring system must be flat but so far it has been impossible to correlate stereochemical changes at particular ring junctions with crystallographic changes, e.g. between rings C and D ( $\alpha$ -ergosterol,  $\beta$ -ergosterol), B and C (lumisterol), A and B (coprostane, cholestane).

(d) *The effect of substituents on the crystallography of the sterols.*

(i) *The position of the hydroxyl group.* A hydroxyl group at  $C_3$  of the sterol skeleton generally, but not invariably leads to double layer formation in the crystal structure and vice versa, a double layer crystal structure usually indicates the presence of a terminal hydroxyl group. Exceptions are cholestane-6-ol which shows a double layer, and pyrocalciferol which does not. Brassicasterol, cerovisterol, the spinasterols and the rubber sterol all probably have hydroxyl groups at  $C_3$ . The position of *i*-cholesterol (which shows one double layer) is obscure.

(ii) *The stereochemistry of the hydroxyl group at  $C_3$ .* It is not possible to distinguish at this stage between the two possible configurations of the hydroxyl group at  $C_3$ . In cholesteryl chloride and bromide, the chlorine and bromine atoms must lie closely in the plane of the ring system.  $\alpha$ -Chlor-cholestane is correlated crystallographically with cholesteryl chloride.

(iii) *The position of the double bonds.* The introduction of either hydroxyl groups or halogens at the double bond system usually produces radical changes in the crystallography which makes simple comparison difficult. The Patterson analysis of cholesteryl chloride hydrochloride provides, however, definite evidence that the extra chlorine atom is at  $C_5$ . The crystallography of both the hydroxy- and maleic anhydride derivatives of ergosterol is in agreement with their present chemical formulae but provides no certain proof of the correctness of these.

(e) *A comparison of the crystallography of different sterols—monohydroxy compounds.* There is a group of sterols including ergosterol and many of the higher plant and animal sterols which show particularly close resemblances in crystal structure to one another. While inclusion in this group must indicate close similarity, both in sterol skeleton and molecular arrangement, deviations do not necessarily seem to have a chemical significance.

(f) *The structure of calciferol.* Calciferol, while showing certain differences from the characteristic sterol group mentioned above, also shows similarities particularly in *c* plane intensities. It seems unlikely therefore that the actual distribution of the atoms in the molecule differs considerably from that in ergosterol.

Many of the outstanding problems of the chemical structure of the sterols can only be settled by exact analysis. The present survey has indicated a number of compounds that are suitable for such treatment.

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**Band spectra of cathodo-luminescence.** By S. T. HENDERSON. (*Communicated by N. F. Mott, F.R.S.—Received 7 July 1939.*)

A photographic method is used to examine the emission bands of various fluorescent materials excited by cathode rays. Single bands given by zinc or zinc-cadmium sulphides agree closely with the Gauss error curve, except at the outer edges where limits to the emission must occur. Approximate graphical analysis gives adequate resolution for more complex bands. Each activator metal produces a band in some characteristic position which varies only slightly with varying preparative methods and activator contents, except when the activator is reduced to very small amounts. The effects of lowering the temperature, and of changing to ultra-violet excitation are described.

A simple relation is demonstrated between band position and composition in the ZnS.CdS series, and the electronic transitions responsible are briefly considered in this case, and also for some manganese-activated materials.

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**The freezing-point of rhodium.** By C. R. BARKER and F. H. SCHOFIELD. (*Communicated by G. W. C. Kaye, F.R.S.—Received 10 July 1939.*)

The freezing-point of rhodium on the International Temperature Scale has been determined by measuring the ratio of brightness, for a certain wave-length, of black-body radiators held at the freezing-points of rhodium and gold, the latter being the basic point of the scale for all high temperatures. The brightness measurements were made by an optical pyrometer of the disappearing-filament type, sighted on the radiator, which, in the case of rhodium, consisted of a closed-end thoria tube projecting into an ingot of the metal. The ingot was contained in a thoria crucible, packed in thoria powder, and placed in an evacuated enclosure. It was heated by electric induction in such a way that it could be alternately melted or frozen at a convenient rate. The ratio of brightness was determined by the successive use of two rotating sectors, the first reducing the brightness of the radiator at the rhodium point so as to give an apparent temperature intermediate between the rhodium and gold points; and the second, when applied to a radiator at this intermediate temperature (about 1270° C), reducing its brightness to that corresponding with the gold point (1063° C). This two-stage reduction in brightness was necessitated by the fact that it is impracticable to cut and measure, with sufficient accuracy, a single sector giving the required ratio of brightness, which is of the order of 700 to 1 for the wave-length used. The purity of the two specimens of rhodium employed in the investigation, was checked by measurement of the ratio of electrical resistance,  $R_{100}/R_0$ .

The value found for the freezing-point,  $1966 \pm 3^\circ \text{C}$ , is indistinguishable from the only previous one obtained by the same method, namely that of the National Bureau of Standards.

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**The  $\lambda 3400$  bands of PH and PD.** By M. ISHAQ and R. W. B. PEARSE.  
(Communicated by A. Fowler, F.R.S.—Received 10 July 1939.)

The band of PD analogous to the  $\lambda 3400$  band of PH has been photographed in the 4th order of a 10 ft. concave grating. An analysis of its rotational structure has been made and the corresponding band-spectrum constants evaluated. In order to compare the values of the constants with those of PH, the PH band has been photographed with the same dispersion and remeasured. The results are:

For the initial  $^3H_i$  state for PD:  $B = 4.175 \text{ cm.}^{-1}$ ;  $D = -1.52 \times 10^{-4} \text{ cm.}^{-1}$ ;  $A = -115.5 \text{ cm.}^{-1}$ ; while for PH:  $B = 8.025 \text{ cm.}^{-1}$ ;  $D = -5.7 \times 10^{-4} \text{ cm.}^{-1}$ ;  $A = -115.5 \text{ cm.}^{-1}$ ; for the final  $^3\Sigma^-$  state for PD:  $B = 4.363 \text{ cm.}^{-1}$ ;  $D = -1.20 \times 10^{-4} \text{ cm.}^{-1}$ ; while for PH:  $B = 8.412 \text{ cm.}^{-1}$ ;  $D = -4.3 \times 10^{-4} \text{ cm.}^{-1}$ .

A catalogue of wave numbers of the lines measured is given, together with their classification.

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**The infra-red absorption spectrum of methylamine vapour.** By C. R. BAILEY, S. C. CARSON and E. F. DALY. (Communicated by C. K. Ingold, F.R.S.—Received 10 July 1939.)

The infra-red absorption spectrum of methylamine vapour has been examined between 1 and  $26.5\mu$  with a prism spectrometer. With the assistance of Raman spectra obtained by other workers fundamental modes and combination tones have been assigned. A model for the methylamine molecule analogous to the  $D_{3h}$  model for ethane has been shown to offer a reasonable explanation of the spectra. With the inclusion of an indirectly determined low torsional frequency in methylamine, the assignment is capable of yielding a satisfactory calculated value for the specific heat.

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**Studies of the post-glacial history of British vegetation. III. Fenland pollen diagrams. IV. Post-glacial changes of relative land- and sea-level in English Fenland.** By H. GODWIN. (*Communicated by Sir Albert Seward, For. Sec. R.S.—Received 26 June 1939.*)

In Parts I and II of this series the author, jointly with Mr Clifford, established an outline of the stratigraphy of the post-glacial deposits of the English Fenland, relating it to archaeology, local vegetational development, and marine transgression and retrogression. Parts III and IV extend these studies by the methods of pollen analysis, in which counting of fossil tree pollen from successive strata is made to convey the history of changing forest cover over a wide land area. Some thirty such sequences of pollen counts have been made, through peat deposits (and sometimes clay) in different parts of the Fenland, and they are presented as pollen diagrams.

Comparison allows the recognition of parallel drifts within these pollen diagrams, which can be divided into a system of zones reflecting phases of forest history. These zones extend from the early period of sparse woodland dominated by birch (Pre-Boreal-zone IV) through the stages of pine-hazel dominance and establishment of alder and mixed oak-forest which continues characteristically to the present day. The latest zone (VIII) is marked by disappearance of the luno, and increased importance of birch, beech and hornbeam. These zones, sometimes subdivided, reflect general climatic drifts of wide extent and permit correlation with forest history, and so in turn with geological events and geochronology on the Continent.

The zoning of the pollen diagrams is shown to confirm strongly the views of Fenland development already derived from stratigraphy, levelling and archaeological content.

Furthermore, by furnishing a time-scale, the pollen-analyses allow a preliminary attempt to co-ordinate in a single quantitative scheme the scattered data of progressive changes in relative land- and sea-level from zone IV onwards. This shows a net marine transgression of about 200 ft., broken by periods of temporary retrogression.

**The decomposition of the alkyl peroxides: dipropyl peroxide, ethyl hydrogen peroxide, propyl hydrogen peroxide.** By E. J. HARRIS. (*Communicated by A. C. G. Egerton, F.R.S.—Received 12 July 1939.*)

The decomposition of dipropyl peroxide like that of diethyl peroxide (*Proc. Roy. Soc. A*, 1938, **168**, 7–18) is a homogeneous, unimolecular reaction below a critical pressure; above this pressure explosive decomposition takes place. The limiting pressure varies with the temperature according to the law for thermal explosions. The products of slow decomposition are complex, and include propaldehyde and an alcohol; the explosive decomposition leads to formation of butane and formaldehyde.

The decomposition of ethyl and propyl hydrogen peroxides is heterogeneous and accelerated by increasing the surface or by coating the surface with salt. The vapours of these peroxides luminesce feebly when admitted to a hot vessel, probably due to combustion of part of the peroxide in oxygen liberated from the rest. The reaction products include aldehydes and alcohols, and at low temperatures oxygen is found, while at high temperatures the hydrocarbon having the same number of carbon atoms as the peroxide is formed.

All the peroxides ignite in air at very low pressures in the temperature range 200–300°, and the connexion between these results and the luminous phenomena of slow combustion is discussed.

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**Hyperfine structure and Zeeman effect of the resonance lines of lithium.** By D. A. JACKSON and H. KUHN. (*Communicated by F. A. Lindemann, F.R.S.—Received 14 July 1939.*)

By means of absorption in atomic beams, it has been possible to observe hyperfine structure in the resonance lines of Li7, and to determine the splitting of the ground state  $2S_{\frac{1}{2}}$  with great accuracy. The value found was  $0.0275 \pm 0.0003 \text{ cm.}^{-1}$ , giving a nuclear magnetic moment  $\mu = 3.25$ .

The hyperfine structure of the  $\pi$ -components of the Zeeman effect of the line  $2S_{\frac{1}{2}}-2^2P_{\frac{1}{2}}$  of Li7 could be resolved. Four lines were observed in each component, in agreement with the value  $I = \frac{3}{2}$  of the nuclear spin.

The multiplet splitting of the  $2^2P$  term of Li7 was found to be  $0.3372 \pm 0.0005 \text{ cm.}^{-1}$ . The isotope shift of the line  $2S_{\frac{1}{2}}-2^2P_{\frac{1}{2}}$  was found to be  $0.345 \text{ cm.}^{-1}$ , and that of the line  $2S_{\frac{1}{2}}-2^2P_{\frac{3}{2}}$  about  $0.36 \text{ cm.}^{-1}$ .

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**Terrestrial heat flow in Great Britain.** By A. E. BENFIELD. (*Communicated by Sir Gerald Lenox-Conyngham, F.R.S.—Received 15 July 1939.*)

Measurements are described which lead to values of the terrestrial heat flow in five bores in Britain. In the two deepest bores definite evidence of the existence of an ice age was found. It is not possible from these measurements, however, to fix the date of the withdrawal of the ice sheet with any certainty, but the indications

are consistent with the estimates derived from other evidence. Using 9000 B.C. as this date to correct the measured heat flows, the mean equilibrium heat flow is found to be  $1.42 \pm 0.09$  cal./cm.<sup>2</sup> sec., which is lower than has been previously estimated, but is higher than the mean heat flow in South Africa found by Bullard. The result of so low a heat flow on temperatures in the granitic and basaltic layers is briefly discussed.

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**Borehole temperatures in the Transvaal and Orange Free State.** By L. J. KRIGE. (*Communicated by B. F. J. Schonland, F.R.S. —Received 15 July 1939.*)

Five deep boreholes in which temperatures were measured pass through dolomite, Ventersdorp lava, Witwatersrand quartzite, intrusive diabase and small thicknesses of other rocks. The geothermic step (increase in depth per °C) varies over wide ranges, its mean values being highest in the dolomite and lowest in the lava. It is, as a rule, small above the water table, especially in the leached dolomite, and generally largest immediately below this level.

In the dolomite below the water table the geothermic step is at first very large and in one case negative, and then decreases almost continuously downward, the lowest value reached being 310 ft. (95 m.) at its base. This decrease and the negative result both seem to be due to circulation of the ground water. The average below the water table is 382 ft. (116 m.) at Gerhardminnebron, 465 ft. (143 m.) at Driefontein and 473 ft. (144 m.) at Doornkloof.

In the Ventersdorp lava in the Jacoba borehole the geothermic step decreases from 345 ft. (105 m.) below the ground water level to 217 ft. (66 m.) near its base, the mean being 248 ft. (76 m.), which is also the average at Doornhoutrivier below the first 1000 ft. (300 m.), where the temperatures are affected by gas.

In the Witwatersrand quartzites at Gerhardminnebron the range is from 470 to 300 ft. (143 to 91 m.) and the average is 366 ft. (111 m.). The value is 380 ft. (116 m.) at Jacoba and 385 ft. (117 m.) at Doornhoutrivier, in both of which holes the thickness of quartzite is small. At Doornkloof the range in the quartzite is from 365 to 335 ft. (111 to 102 m.) and the average 354 ft. (108 m.), which is also the mean value in two dykes passing through the quartzite. This agreement seems to show that the geothermic step in the quartzite is reduced by the presence of the dykes, and vice versa.

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**Heat flow in South Africa.** By E. C. BULLARD. (*Communicated by B. F. J. Schonland, F.R.S. —Received 15 July 1939.*)

The thermal conductivities of forty-nine rocks from bores in South Africa have been measured. The mean heat flow calculated from these and from Krige's and Weiss's temperature measurements is  $1.18 \times 10^{-8}$  cal./cm.<sup>2</sup> sec. This is much lower than the values usually quoted but agrees with Benfield's mean for bores in Europe. There is no foundation for the common opinion that the heat flow is lower in South

Africa than in Europe. It is likely that many of the other supposed cases of abnormal heat flow are also unfounded. The low value of the heat flow shows that there cannot be more than 12 km. of rock with the radioactivity of surface granite under South Africa. This supports the reality of the "Intermediate Layer" of seismology and leads to very low temperatures at the base of the granitic layer.

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**The exchange reaction between deuterium and hydrogen chloride. I. By E. K. RIDEAL, F.R.S. and H. STEINER. (Received 20 July 1939.)**

The thermal exchange reactions between the following substances have been studied: deuterium and hydrogen chloride, hydrogen and deuterium chloride, *p*-hydrogen and hydrogen chloride. The mechanism of these reactions consists of a superposition of a bimolecular reaction of the type  $D_2 + HCl \rightarrow HD + DCl$ , and of an atomic chain reaction, initiated by thermally dissociated atoms. The atomic chain is found to proceed by the sequence  $D + HCl \rightarrow HD + Cl$ ,  $Cl + D_2 \rightarrow D + DCl$ , while the reaction  $D + HCl \rightarrow DCl + H$  does not occur.

Activation energies, calculated from band spectroscopic data by Eyring and Polanyi's method, are in agreement with the experimental results, in that they show that both these mechanisms can occur. From the form of a potential energy contour-map the non-occurrence of the reaction  $D + HCl \rightarrow DCl + H$  can be explained as due to a low transmission coefficient. In addition to these results, values for the rate of some of the elementary reactions involved have been obtained and are discussed in the light of the transition state theory.

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**The exchange reaction between deuterium and hydrogen bromide. By H. STEINER. (Communicated by E. K. Rideal, F.R.S.—Received 20 July 1939.)**

The exchange reactions of para-hydrogen and of deuterium with hydrogen bromide have been studied. It was found that the mechanism differs considerably from the one of the analogous reaction between deuterium and hydrogen chloride. No bimolecular reaction was found and the atomic chain reaction, which caused the exchange, followed the alternative of the two possible sequences. Potential energy diagrams were constructed from band spectroscopic data according to the methods developed by Eyring and Polanyi. They were found to account satisfactorily for the changes observed. In addition, values for the rate of the reaction  $H + HBr \rightarrow H_2 + Br$  are given and discussed. The transition state theory seems to account somewhat better than the collision theory for the temperature dependence of the rate of this reaction.

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**The adsorption of non-polar gases on alkali halide crystals.** By W. J. C. ORR. (*Communicated by E. K. Rideal, F.R.S.—Received 20 July 1939.*)

An experimental technique sufficiently sensitive to measure accurately the very small amounts of non-polar gases which are adsorbed on alkali halide crystals is developed. Isotherms for argon, oxygen and nitrogen on KCl and CsI are described and the heats of adsorption as a function of the amount adsorbed are derived.

For these systems a maximum occurs in the curve of the heat of adsorption plotted as a function of the amount adsorbed and this, it is shown, marks precisely the position at which a first monolayer is completed. The results are compared with theoretical calculations of the adsorption behaviour of argon on KCl and CsI and very good general agreement is obtained. Probable explanations are advanced for specific deviations.

In the range between the completion of a single monolayer and the bulk condensation of the gas an amount equal to three to four times that in the first layer is adsorbed. It is concluded that the molecules in this region tend to arrange themselves in layers rather than in localized aggregates.

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**$\gamma$ -carotene in the sexual phase of the aquatic fungus *Allomyces*.** By R. EMERSON and D. L. FOX. (*Communicated by Sir Frederick Gowland Hopkins, F.R.S. —Received 20 July 1939.*)

In the life-cycle of certain species of the aquatic phycomycoto *Allomyces* an asexual or sporophytic generation regularly alternates with a sexual or gametophytic one. Asexual plants bear thin-walled, colourless zoosporangia and thick-walled, brown, resistant sporangia; sexual plants bear colourless female gametangia and orange male gametangia.

The pigment in the resistant sporangia is confined to the heavy, pitted, outer wall; the inner membrane, the cytoplasm, and the spores which are formed are all colourless. The orange pigment, found exclusively in the male cells of the sexual phase, is present in oil-droplets within the cytoplasm and is still apparent in the male gametes after their emergence from the gametangia. There is not any trace of pigment in the female gametes, which, although more than twice the size of the males, are similar to the latter in structure and motility.

Results of the present chemical investigation of the pigments in *Allomyces* may be summarized as follows. The brown pigment of the resistant sporangia belongs to the melanin group, and careful examination and tests have failed to reveal any detectable traces of carotenoids in the solvent-free material extracted from sporophytic plants. The brilliant orange colour of the male cells in the sexual generation, on the other hand, is clearly due almost entirely, in the majority of cases examined, to the presence of carotenoid pigments. Melanins are rare or lacking in this sexual phase. No traces of oxygen-containing carotenoids, i.e. xanthophylls, xanthophyll-esters, or carotenoid acids have been detected.  $\gamma$ -carotene was found in remarkably high concentrations, accompanied in certain strains by small amounts of other

isomers such as  $\beta$ -carotene. The pronounced and nearly exclusive synthesis of the relatively rare  $\gamma$ -isomer of carotene is of particular interest.

These findings concerning the pigmentation of *Allomyces* are in close accord with existing information: the synthesis and selective storage of carotenoids or their derivatives in structures associated with reproduction is well known in many other cryptogams as well as in countless higher plants. Notable also is the corresponding concentration of carotenoid pigments in the reproductive structures and secretions of many animals. The foregoing study lends emphasis to the possibility that such compounds play important biochemical roles in sexuality and the processes involved in the metabolism of reproduction.

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**The upper frequency limit for the binaural localization of a pure tone by phase difference.** By J. W. HUGHES. (*Communicated by T. Graham Brown, F.R.S.—Received 20 July 1939.*)

The least perceptible changes of phase in binaural localization (measured from zero phase difference) have been determined for several observers at various frequencies in an endeavour to find if any upper frequency limit for the detection of such changes existed. Pure tones were used in the experiments, and the changes of phase were made by an electrical method. The results show that for nearly all the observers the detection of changes of phase becomes difficult at about 1300 c./sec., and impossible at about 1500 c./sec.

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**Triassic inflorescences from South Africa and their significance in the floral morphology of the angiosperms.** By H. HAMSHAW THOMAS, F.R.S. (*Received 22 July 1939.*)

Two interesting specimens found by Dr A. L. DuToit in the Molteno Beds near Dordrecht, C.P., are described and figured. They were preserved as impressions in mudstone. One specimen has the appearance of a branched angiospermous inflorescence bearing small flowers and buds; the branches lie in one plane. The flower-like structures had five or six perianth segments surrounding a flattened receptacle or shallow cup. The central structures are so altered by compression that their nature cannot be ascertained, they must have been small and sessile. The other specimen shows two "flowers" with a perianth and some different structures, possibly seed bearing. Its branches bear bracts and bracteoles.

A new genus is founded for the specimens which are regarded as specifically distinct. Their affinities are unknown. While showing some resemblance to modern angiosperms, these fossils have no trace of strobilar construction. They raise doubts as to the validity of the commonly held views of floral morphology.

A critical analysis of the discussions on floral evolution shows disregard for the rules of logic and resulting confusion. Evolutionary concepts must be clearly separated from those of phylogeny.



An inductive approach to the question of the strobilar theory of floral construction is suggested. The significance of these specimens in relation to this enquiry and to the recent investigations of Grégoire on floral ontogeny is indicated.

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**The magnetic susceptibilities of some cupric salts.** By JAMES REEKIE.  
(Communicated by J. D. Cockcroft, F.R.S.—Received 22 July 1939.)

The mean mass susceptibilities of the three cupric salts—copper sulphate pentahydrate, copper potassium sulphate hexahydrate, and copper ammonium sulphate hexahydrate—have been measured down to a temperature of 1.6° K. It is found that the first salt follows approximately a Weiss law with  $\theta = -0.7^\circ$  over the whole temperature range, and that both double sulphates follow a similar law with  $\theta = -0.3^\circ$  down to 14° K but obey a simple Curie law in the helium region. The results are compared with Jordahl's crystalline field theory in which the  $\text{Cu}^{++}$  ion in these salts is assumed to be acted upon by an electrostatic field of nearly cubic symmetry.

It is concluded that Jordahl's theory is able satisfactorily to account for the observed variation with temperature of the effective magnetic moment of the  $\text{Cu}^{++}$  ion in the double sulphates, but that in the case of the copper sulphate the theory is not adequate to explain the susceptibility measurements. It appears that either the system of energy levels of the  $\text{Cu}^{++}$  ion in copper sulphate must differ considerably from that in the double sulphates, or magnetic interaction effects must become appreciable in copper sulphate at a relatively high temperature.

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**A rigorous theory of the recombination of both small and large ions in gases at high pressures.** By W. R. HARPER. (Communicated by A. M. Tyndall, F.R.S.—Received 22 July 1939.)

The recombination coefficient for both large and small ions at high pressures is derived by a rigorous treatment. For small ions the formula reduces to that of Langevin. It is shown how it was possible for Langevin to obtain the correct result without allowing for the effects of diffusion. The condition for the recombination coefficient to be independent of concentration is investigated, and it is found that for small ions the theory is not invalidated on this account unless concentrations are greatly in excess of those found in an  $\alpha$ -particle track at 100 atm.

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**The thermal conductivity of gases by a relative method with an application to deuterium.** By W. G. KANNULUIK. (Communicated by T. H. Laby, F.R.S.—Received 24 July 1939.)

By means of a hot-wire method in which short thick wires are used the thermal conductivity of a gas relative to that of hydrogen can be determined to 0.5%. If the value of the conductivity of hydrogen be assumed, the thermal conductivity of any other gas can be obtained in absolute measure correct to 1%.

The method is based on a simple approximate theory applicable to a short thick wire mounted axially in a metal tube. It is shown that for a given heating current

$$1 \text{ amp. in the wire } k = \frac{C}{R - R_0} + C',$$

where  $k$  is conductivity of the gas filling the tube;  $R$  is the mean resistance of the heated wire;  $R_0$  is true resistance at  $0^\circ\text{C}$ ;  $C$  and  $C'$  are specific constants, the determination of which calibrates the apparatus.

The apparatus consists of two geometrically similar stainless steel tubes  $R$  and  $S$  each containing a nickel wire 5 cm. long and 1.5 mm. in diameter. The two tubes form the low-resistance arms of a Kelvin bridge while two pairs of 1 ohm coils comprise the ratio arms. One coil in each ratio arm is suitably shunted in order to balance the bridge. The tube  $S$  is left permanently filled with air while  $R$  is filled with any gas under investigation.

The constants  $C$  and  $C'$  are determined by obtaining  $(R - R_0)$  for hydrogen ( $k = 41.3 \times 10^{-8}$  at  $0^\circ\text{C}$ ) and  $(R - R_0)$  with  $R$  highly evaluated ( $k = 0$ ). Satisfactory checks on the method were then obtained by measuring the conductivity of air and of carbon dioxide.

The tube  $R$  was next filled with pure deuterium obtained from 99.95% heavy water by reducing the latter over heated magnesium ribbon. At  $0^\circ\text{C}$  the ratio of the conductivity of hydrogen to that of deuterium was found to be 1.365, while the conductivity of  $D_2$  in absolute measure was  $30.3 \times 10^{-8}$  cal. cm.<sup>-1</sup> sec.<sup>-1</sup> deg.<sup>-1</sup>.

**Investigations on the iodide of sulphur. Parts I and II.** By M. R. ASWATHANAVAYANA RAO. (*Communicated by Sir Martin Forster, F.R.S.—Received 24 July 1939.*)

#### Part I

In chemical literature, sulphur iodide has been stated to have no existence. In this paper evidence is presented to show that sulphur monoiodide is formed in carbon tetrachloride solutions, when a dilute solution of sulphur chloride is treated with solid potassium iodide.

A dilute solution of sulphur iodide is yellow in colour. The iodide rapidly decomposes at ordinary temperatures, giving sulphur and iodine. At low temperatures, however, the solutions are comparatively stable. Light is found to promote the decomposition of sulphur iodide.

The reaction of sulphur iodide (in carbon tetrachloride solutions) with aqueous sodium hydroxide is analogous to that of sulphur chloride with alkali.

#### Part II

The velocity of decomposition of sulphur iodide in carbon tetrachloride solution has been studied at  $0^\circ$  and at  $30^\circ$ . At  $30^\circ$ , the velocity of decomposition is four times that at  $0^\circ\text{C}$ .

With sulphur iodide solutions, a complete absorption for all wave-lengths below  $4770 \text{ \AA}$  is noticed. Data on the instability of sulphur iodide obtained by the analytical methods are confirmed by the spectroscopic investigations.

# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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**Adsorption of lauryl sulphonic acid in the surface of its aqueous solution, and the Gibbs theorem.** By J. W. MCBAIN, F.R.S. and LL. A. WOOD. (*Received 25 July 1939.*)

One hundred and seventy-two measurements made with the microtome and with the interferometer methods agree in showing that positive adsorption occurs in the surface of all dilute solutions of aqueous lauryl sulphonic acid. A similar result follows from dynamic measurements and from measurements with sodium butyl benzene sulphonate and butyl benzene sulphonic acid. No time effect is observable after the first few minutes.

The surface tension of solutions of lauryl sulphonic acid changes over long periods of time extending to hours or days.

Since the surface tension is at a minimum in 0.0062 N lauryl sulphonic acid, the Gibbs theorem would predict zero adsorption followed by negative adsorption. Similar predictions would follow for all the numerous instances of type III curves where in extreme dilution the surface tension is lowered to a fraction of that of water, thereafter passing through a minimum or remaining constant.

It is suggested that the initial equation in the Gibbs derivation does not completely enumerate all the forms of energy actually involved.

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**The reaction between type II *Pneumococcus* antiserum and a glucuronide azo-protein.** By B. WOOLF. (*Communicated by J. B. S. Haldane, F.R.S.—Received 26 July 1939.*)

An amino derivative of the natural glucuronide, euxanthic acid, was prepared and coupled by the azo-link to proteins.

These azo-proteins gave precipitates with type II *Pneumococcus* antiserum, which were inhibited by glucuronides. The effect of varying azo-protein/serum ratio on the amount and composition of the precipitate was similar to the effect of varying polysaccharide/ratio on the precipitate formed when the specific polysaccharide is added to the serum.

Attempts to immunize rabbits and mice with amino-euxanthic acid azo-protein were not successful.

Chemical estimations and animal protection experiments indicated that the material precipitated by the azo-protein from the serum was part of the antibody to type II *Pneumococcus*.

These results support the view that at any rate part of the specificity of type II serum for the type polysaccharide is due to glucuronic acid or some closely similar grouping.

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**Quantitative studies on the effect of haptenes on the reaction between antigens and antibodies.** By B. WOOLF. (*Communicated by J. B. S. Haldane, F.R.S.*—Received 26 July 1939.)

Quantitative studies on the inhibition by haptenes of the antigen-antibody precipitate have been made on three systems: (i) *Pneumococcus* type II polysaccharide and type II antiserum, with sodium euxanthate as inhibitor; (ii) partially hydrolysed cherry gum and type II *Pneumococcus* antiserum, with sodium euxanthate as inhibitor; (iii) atoxyl-azo-egg albumin and atoxyl-azo-globulin antiserum, with atoxyl as inhibitor.

In all three systems the inhibition by a given concentration of haptene varied very little over a wide range of antigen/antibody ratios. While the amount of precipitate depends mainly on the total amounts of antigen and antibody, varying little with dilution, the degree of inhibition depends on the concentration and not the total amount of haptene.

In system (i) there was only partial inhibition even with high concentrations of haptene. The concentration of haptene required to give half the maximum effect was about 16 times as great as in system (ii). In system (iii), the ratio of antibody to azo-protein in the precipitate is lowered by the presence of haptene. There are wide variations in sera from different rabbits in the concentration of haptene required to give the same degree of inhibition.

The bearing of these results on the nature of the action between antigen and antibody is discussed.

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**The molecular fields of carbon dioxide and nitrous oxide.** By R. E. BASTICK, H. R. HEATH and T. L. IBBS. (*Communicated by M. L. Oliphant, F.R.S.*—Received 28 July 1939.)

Measurements of thermal diffusion in mixtures containing nitrous oxide have been made between room temperature and about 300° C, which show that there is a sharp change in the nature of the molecular field of this gas at about 120° C. This conclusion

is supported by viscosity measurements. Further thermal diffusion experiments have been made on carbon dioxide which support our previous conclusion that there is a similar anomaly in the field at about 145° C.

**Experiments on the transmutation of fluorine by deuterons.** By J. C. BOWER and W. E. BURCHAM. (*Communicated by J. D. Cockcroft, F.R.S.—Received 28 July 1939.*)

The emission of protons from fluorine under bombardment by deuterons of  $0.8 \times 10^6$  eV energy has been studied by means of an expansion chamber. Five groups of protons of ranges 13.0, 18.8, 21.0, 24.2 and 31.0 cm. have been found and from the energy values of these groups it is concluded that excitation levels at 0.7, 1.0, 1.35 and  $1.9 \times 10^6$  eV above the ground state exist in the radioactive  $^{20}\text{F}$  nucleus. From the energy of the 31 cm. group a value of 20.0063 is deduced for the mass of radioactive  $^{20}\text{F}$ . An energy release of  $7.0 \times 10^6$  eV is therefore to be expected in the  $\beta$  decay of  $^{20}\text{F}$  to  $^{20}\text{Ne}$ , and the disagreement between this value and the observed  $\beta$ -ray end point of  $5.2 \times 10^6$  eV has been partially reconciled by the observation that a radioactive emission of  $\gamma$ -rays of  $2.2 \times 10^6$  eV energy accompanies the  $\beta$  emission. The origin of this  $\gamma$  radiation is discussed in terms of the level system of  $^{20}\text{Ne}$ . It is shown that the experimental results do not support the original theory of Bethe, Hoyle and Peierls concerning the complexity of the  $\beta$ -ray spectrum of  $^{20}\text{F}$ .

A new transmutation of the carbon isotope  $^{13}\text{C}$  by deuterons has been detected.

**The equilibrium of grains as they lie on the bed of a flowing stream.** By C. M. WHITE. (*Communicated by G. I. Taylor, F.R.S.—Received 1 August 1939.*)

Local turbulence, large scale turbulence, the packing of the grains, and their angle of surface repose are shown to be important variables.

New kinds of experiments to separate the several actions are described.

Rounded sand grains, subjected to the steady drag of a viscous fluid applied to their upper parts begin to move when

$$\tau = 0.18\rho'gk \tan \phi,$$

when  $\tau \equiv \rho V_*^2$  is the average shear stress to start motion,  $\rho'$  is the density of the grain less that of the fluid,  $k$  is the grain diameter, and  $\phi$  is the angle of surface repose. This would be valid also if the force were steadily applied by an inviscid fluid in the particular case when, as they usually are, the centres of grains in the top layer are  $1.75k$  apart.

Wall turbulence, and the large scale turbulence of parallel channels, each halve the above value; acting together they divide it by four. In diverging channels, large scale turbulence may move the grains when the average drag is zero: in converging channels the extra drag is offset by the absence of large scale turbulence. The experiments extended over the range  $0.04 < V_*k/\nu < 1300$ . In an appendix the drag of flat plates is calculated and compared with an experiment for certain simple boundary layer conditions.

**The repulsive forces between isotopic molecules.** By R. P. BELL. (*Communicated by C. N. Hinshelwood, F.R.S.—Received 2 August 1939.*)

It is pointed out that the charge distribution in a diatomic molecule will be a function of the zero-point energy of the nuclear vibration, and hence that the forces of attraction and repulsion between two such molecules will depend on the nuclear masses. In the case of the Coulomb repulsion the magnitude of this effect is estimated by the quantitative treatment of a simple model. Calculated numerical data are given which indicate that this effect of the nuclear mass should lead to a measurable difference between the second virial coefficients of hydrogen and deuterium, though there are several other factors which would also contribute to the observed difference.

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**The lipochromes and sterols of the algal classes.** By P. W. CARTER, I. M. HEILBRON, F.R.S. and B. LYTHGOE. (*Received 4 August 1939.*)

An examination has been made of the specific lipochrome pigments and sterols present in representative members from seven of the eleven classes of the algae. Fresh material, free from symbiotic and epiphytic algae, has been used throughout. The uniformity of pigmentation implied in the classification of the algae is borne out by the present work, this being most marked in the Phaeophyceae. The higher orders of this class differ from all other classes of algae in the absence of xanthophyll and without exception synthesize carotene and fucoxanthin. The presence of fucosterol alone is also characteristic of the class. The Chlorophyceae resemble the higher plants closely in their synthesis of carotenoid pigments and sterols, but here *Zygnema pectinatum* is exceptional in synthesizing fucoxanthin and *Vaucheria hamata* in forming violaxanthin. Chemically the Bacillariophyceae and the Chrysophyceae are found to show a close relationship to the more primitive orders of the Phaeophyceae while the Xanthophyceae approximate to the Chlorophyceae. The Rhodophyceae all contain carotene and lutein in addition to the water soluble chromoproteins, but in the primitive orders there is no evidence of the presence of the epiphyasic myxoxanthin which is characteristic of the Myxophyceae. *Polysiphonia nigrescens* is an exceptional red alga in synthesizing fucoxanthin (and fucosterol) in addition to carotene and lutein. The absence of sterols from the Myxophyceae may be correlated with the lack of sexuality in the class.

No habitat differences could be detected nor any variation in the haploid and diploid phases of the life-cycle.

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**The  $\beta$ -radiations of uranium  $X_1$  and uranium  $X_2$ .** By J. S. MARSHALL. (*Communicated by W. L. Bragg, F.R.S.—Received 5 August 1939.*)

The distribution with momentum of the  $\beta$ -rays of uranium  $X$  has been investigated, first using a cloud chamber with particular regard to momentum values below  $H\rho$  2000 (280 keV), and then using a magnetic spectrometer to determine the distribution above that value.

Evidence has been obtained that the  $\beta$ -rays of  $UX_1$  extend at least to  $H\mu$  1950 (265 keV). This value taken as an end-point gives a point on the Sargent diagram well to the right of the curve of allowed transitions. This fact suggests interpretation in terms of partial spectra, an interpretation to which the shape of the cloud chamber distribution is amenable. Fermi distributions with end-points at 130 and 300 keV ( $H\mu$  1300 and 2100) and areas in the ratio 4 to 5 have been fitted.

The spectrometer measurements have revealed a  $\beta$ -ray line at  $H\mu$  3600 (686 keV), intensity  $0.0036 \pm 0.0004$  per disintegration, indicating the presence of a  $\beta$ -ray of energy 802 keV. From unpublished measurements by J. A. Gray, the intensity of this  $\beta$ -ray has been taken tentatively as 0.05 quanta per disintegration. With this supporting evidence, the continuous distribution for the  $UX_2$   $\beta$ -rays is interpreted, above  $H\mu$  3000 (525 keV), as formed by two partial spectra of the Fermi type, one containing about 95 % of the intensity and extending to  $H\mu$  9300 (2.32 MeV, this value being taken from Ward and Gray 1937), and the other with about 5 % of the total intensity, extending to  $H\mu$  6550 (1.52 MeV). The  $\gamma$ -ray intensity and the strength of the  $\beta$ -ray line give an internal conversion coefficient for the  $\gamma$ -ray of about 0.07.

The energy of the  $\gamma$ -ray agrees sufficiently well with the energy of one of the  $\gamma$ -rays of  $UZ$ , according to Feather and Bretscher (1938). This constitutes added evidence for the isomerism of the nuclei  $UX_2$  and  $UZ$ .

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#### Auger and secondary X-ray electrons from gold. By R. L. MAYO and H. R. ROBINSON, F.R.S. (Received 9 August 1939.)

Measurements have been made, in the magnetic spectrograph, of the energies and approximate relative intensities of the Auger electrons emitted by gold atoms which have been ionized in their  $L$  levels. Material is obtained for a direct comparison of these energies with the energies of the X-ray electrons expelled by primary gold radiations from normal gold atoms. The analysis is however incomplete, and more powerful methods will be required to elucidate the complete Auger spectrum.

A new value of  $h/e$  has been deduced from the results of the measurements on the X-ray electron groups.

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#### Development *in vitro* of the mammalian gonad. II. The effect of subnormal temperatures on the differentiation and survival of embryonic and infantile rat and mouse ovaries. By P. N. MARTINOVITCH. (Communicated by F. H. A. Marshall, F.R.S.—Received 10 August 1939.)

The effect of cultivating rat and mouse ovaries at subnormal temperatures has been studied.

The explanted ovaries thrive much better at  $34^\circ\text{C}$  than at  $37^\circ\text{C}$ . They survive longer, differentiate better and show less necrosis. Thus infantile mouse ovaries incubated at  $34^\circ\text{C}$  survived for as long as 80 days and germ cells persisted throughout this period. Control cultures incubated at  $37^\circ\text{C}$  never survived longer than 30-40

days. During the first two months' cultivation at 34° C, relays of germ cells enlarged, attained the normal maximum size and sometimes passed into the metaphase of the first maturation division.

Infantile rat ovaries incubated at 34° C remained healthy after 103 days *in vitro* and contained normal residual ova at the end of this period, whilst controls at 37° C degenerated after 60–70 days.

Embryonic mouse ovaries incubated at 32° C developed less well and showed more degeneration than those kept at 34° C, indicating that the optimum temperature for the survival of mouse ovaries *in vitro* is about 34° C.

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**The suppression of meiosis and the origin of diplochromosomes.** By H. N. BARBER. (*Communicated by Sir Daniel Hall, F.R.S.—Received 11 August 1939.*)

Heating after pachytene has no effect on the frequency or proximal localization of chiasmata in the pollen mother-cells of *Fritillaria meleagris*.

Continued high temperatures during the meiotic divisions have two effects: (a) the separation of the chromosomes is followed by the development of a restitution nucleus giving diploid or tetraploid pollen grains with normal chromosomes; (b) the meiotic nuclear divisions are completely suppressed, so that a diplotene nucleus lapses directly into a pollen grain resting nucleus. The pollen grain chromatid division takes place to give, at the metaphase of the pollen grain mitosis, diplochromosome bivalents consisting of eight chromatids.

The diplobivalents retain the chiasma structure of the meiotic bivalents except two chromatids replace one.

Each chromatid in a diplobivalent has a simple mitotic coil and is coiled independently of the others. There is no well-defined attraction in pairs, each arm consisting of two parallel chromatids at full metaphase. Thus joint coiling of the major spirals is a condition of the retention of the arrangement of chromatids in pairs at first metaphase of meiosis.

Each half diplobivalent has a single centromere with the four chromatids passing through it. The centromeres orientate separately. Thus the co-orientation of centromeres at meiosis depends on the internal structure of the centromere, not on chiasma formation.

At anaphase two successive divisions take place in the centromere. The first separates the products of the pachytene division; the second the products of the pollen grain resting stage division. The mitosis will thus give two tetraploid daughter-nuclei with normal chromosomes. They will differ by the segregation of the products of crossing over at the suppressed meiosis.

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**The friction of lubricated metals.** By F. P. BOWDEN and L. LEBEN. (*Communicated by G. I. Taylor, F.R.S.—Received 11 August 1939.*)

An analysis has been made of the kinetic friction between metals sliding under conditions of boundary lubrication. With mineral oils and many other lubricants an



intermittent clutching and breaking away of the surfaces still occurs through the oil film. The friction, the surface temperature and the area of contact all show violent fluctuations and the behaviour may be essentially the same as with unlubricated metals. Certain substances, however, are able to prevent this "stick-slip" motion and allow continuous sliding to take place. With short chain fatty acids, for example, the motion is "stick-slip", but when the chain reaches a certain length continuous sliding occurs. Alcohols and saturated hydrocarbons of the same, or longer, chain length do not cause continuous sliding. Even with the best lubricant the film breaks down to some extent during sliding and some wear of the surfaces takes place. The metal is torn to a depth which is large compared with the dimensions of a molecule. The frictional force between lubricated metals must therefore be greatly influenced by the bulk properties of the metals concerned.

The frictional behaviour of metallic surfaces covered with successive monolayers of lubricant has also been investigated. A single layer can cause a large reduction in the friction but the film is soon worn away. With multilayers the rate at which the film wears off is markedly dependent on its molecular thickness and methods are described for measuring the rate of wear of lubricant films. A single film of long chain fatty acid molecules is more effective than a single film of the flat leaf-shaped cholesterol molecule.

It is clear that a primary film is not sufficient, but that for effective boundary lubrication it is necessary to have present a layer of lubricant several molecules thick. The experiments show that boundary lubrication cannot be regarded as a purely surface phenomenon.

On the basis of those experiments a theory has been put forward to explain boundary lubrication. In general it appears that even with lubricated surfaces the local pressures in the region of contact are very high, so that the lubricant film between the surfaces is partly broken down. If the sliding speeds are appreciable, this breakdown is aided by the local high temperature. Metallic junctions, the size of which is large compared with the dimensions of a molecule, are formed between the surfaces. There will of course be some resistance due to the interaction of the surface films themselves but, under many conditions of sliding, the resistance to motion is due mainly to the force necessary to break the junctions. The frictional behaviour of boundary lubricated surfaces is therefore largely governed by the extent to which the lubricant film breaks down during sliding.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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25 OCTOBER 1939

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**The scattering of mesotrons in metal plates.** By J. G. WILSON. (*Communicated by P. M. S. Blackett, F.R.S.—Received 14 August 1939.*)

An extended series of accurate measurements of the scattering of mesotrons is described. These are in general agreement with our earlier measurements, and confirm within the accuracy of the experiment (about 4 %) the theoretical value given by Williams (1939) for multiple Coulomb scattering.

Theory shows that the scattering by a finite nucleus should be about 10 % less than that by a point nucleus. The experimental results, which have an accuracy of about 4 %, definitely confirm the lower value and so can be considered as giving evidence as to the correctness of the scattering theory as applied to a finite nucleus.

The evidence for the existence of large-angle scattering due to short-range forces between mesotrons and nuclear heavy particles (protons and neutrons) is discussed. The cross-section for this type of scattering is estimated to be of the order  $10^{-28}$  cm.<sup>2</sup>, and this value is in agreement with that given by Bhabha for a "classical" mesotron theory.

There is no experimental evidence for the large increase of scattering due to short-range forces at low mesotron energies given in the quantum mechanical treatment due to Heitler. For the available mesotrons of lowest energy, the cross-section is found to be less than  $10^{-27}$  cm.<sup>2</sup>. This result is not compatible with the present development of mesotron theory, and may be interpreted as indicating a failure in the treatment of the charge exchange which leads to the interaction between charged mesotrons and heavy particles.

**The photodisintegration of the deuteron in the meson theory.** By H. FRÖHLICH, W. HEITLER and B. KAHN. (*Communicated by J. D. Cockcroft, F.R.S.—Received 14 August 1939.*)

We have calculated the photoelectric effect of the deuteron on grounds of the meson theory of nuclear forces. In this theory the nuclear field is considered as electrically charged, and consequently the light quantum can act on the nuclear field as well as on the proton. Because of the small mass of the meson, this effect is large. For the calculation we have assumed for the ground state of the deuteron a  $^1S$  state. The result is that at high frequencies  $\nu$  the cross-section is much larger than in the old theory, and decreases only like  $1/\nu^4$  as compared with the  $1/\nu^6$  law in the Bethe-Peierls theory. Also the angular distribution is different. In addition to the  $\sin^2\theta$  distribution about the direction of the  $\gamma$ -ray, there is a  $\theta$ -independent term which becomes increasingly important for increasing energies. For the Li  $\gamma$ -rays (17 MeV), for instance, the cross-section is seven times larger than in the old theory and the ratio of the number of protons emitted parallel to the  $\gamma$ -ray to that emitted perpendicular to it is 0.7.

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**The absorption spectra of ethylene, deuterio-ethylene and some alkyl-substituted ethylenes in the vacuum ultra-violet.** By W. C. PRICE and W. T. TUTTE. (*Communicated by R. G. W. Norrish, F.R.S.—Received 14 August 1939.*)

The absorption spectra of  $C_2H_4$ ,  $C_2D_4$  and  $C_2H_3D$  have been investigated in the region 2000–1000 Å. The spectra are interpreted as arising from the excitation and photoionization of the " $\pi$ " electrons of the double bond. The ionization potential obtained by the extrapolation of successive excited states in Rydberg series is 10.45 V probably accurate to 0.03 V. The spectra of (a) propylene, (b) butene-2- (trans) and cyclohexene, (c) trimethyl ethylene, (d) tetramethyl ethylene, are shifted progressively to long wavelengths corresponding to reduction of their ionization potentials to (a) 9.6 V, (b) 9.2 V, (c)  $\sim 8.75$  V and (d)  $\sim 8.3$  V. This is probably a charge transfer or inductive effect brought about by the additional alkyl groups.

The spectrum of *cis*-dichloro-ethylene is discussed and its low ionization potential of 9.6 V is attributed to resonance to ionic states similar to those postulated to explain the ortho-para substituting properties in the phenyl halides. The value 11.3 V obtained for trans-dichloroethylene by Mahneke and Noyes is attributed to a non-bonding " $\pi\pi$ " Cl electron.

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**The absorption spectra of conjugated dienes in the vacuum ultra-violet.** By W. C. PRICE and A. D. WALSH. (*Communicated by R. G. W. Norrish, F.R.S.—Received 14 August 1939.*)

The absorption spectra of butadiene, isoprene,  $\beta\gamma$ -dimethyl butadiene and chloroprene have been investigated in the region 2500–1000 Å. They are all very similar. The first strong bands appear as short diffuse progressions involving the  $C=C$

vibrational frequency but no vibrational structure is observed in the bands below 1800 Å. The latter are interpreted as vibrationless electronic transitions converging to the first photoionization limit  $\chi_1^2\chi_1$  of the "mobile" electrons. The extrapolation of successively excited states in Rydberg series gives the following very accurate values of the molecular ionization potentials:—butadiene: 9.02 V; isoprene: 8.81 V;  $\beta\gamma$ -dimethyl butadiene: 8.67 V; chloroprene: 8.79 V.

The diminution in the ionization potential with alkyl substitution is interpreted as a simple inductive effect. The diminution in chloroprene involves the mesomeric as well as the inductive effect. The non-appearance of vibration bands accompanying the Rydberg transitions is a direct result of the resonance. As the electron is shared between two bonds, its removal has only one-half the effect on each and this is apparently inadequate to cause the appearance of vibrational bands. Attempts are made to evaluate the resonance integral from various features of the spectra and from the ionization potentials. It is pointed out that the main difficulty with Mulliken's  $N \rightarrow V$  interpretation of the strong bands of the dienes (also that of Sklar's for benzene, etc.) is that the shorter wavelength transitions are interpreted as being the more strongly antibonding. The reverse is observed to be the case as judged from the vibration frequencies appearing in the spectra.

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**Investigations of infra-red spectra (2.5–7.5 $\mu$ )—absorption of water. By J. J. FOX and A. E. MARTIN. (Communicated by Sir Robert Robertson, F.R.S.—Received 15 August 1939.)**

The absorption spectrum of water in the form of gas, liquid, solid, and in solution in carbon tetrachloride has been investigated in the region 2.5–7.5  $\mu$ .

In the liquid and solid the bands at 3, 4.7, and 6  $\mu$  have been studied, and their variation with temperature in the liquid determined. The 3  $\mu$  band moves to higher frequencies as the temperature increases, while the 4.7 and 6  $\mu$  bands move in the opposite direction. This behaviour is attributed to hydrogen bonds between water molecules reducing the OH valency frequencies but increasing the angular deformation frequency by reason of the constraints imposed by the surrounding molecules. Since the hydrogen bonds are at their strongest in ice and become progressively weaker when ice melts and the temperature of the water is raised, we expect to find the frequencies in the order ice-water at 0° C, water at 100° C, vapour in accordance with the observations. The 4.7  $\mu$  band is a combination of the deformation frequency and the frequency of hindered rotation  $\nu_R$ , about 500  $\text{cm}^{-1}$ , which has previously been observed both in the Raman and infra-red spectra; its large temperature variation is due to the rapid diminution of  $\nu_R$  with rise of temperature until finally it vanishes in the vapour state.

The absorption of water in carbon tetrachloride about 2.7  $\mu$  has been studied and the results of Rodebush *et al.* confirmed, except that the suggestion of rotational structure reported by these workers could not be substantiated. Our values for the valency vibration frequencies are 3705 and 3641  $\text{cm}^{-1}$ , and are 40–50  $\text{cm}^{-1}$ , less than the corresponding vapour values. A rapid method of eliminating water from

carbon tetrachloride (and some other solvents and solutions) is described, and a value obtained for the solubility of water in carbon tetrachloride.

Water vapour was examined in the region of  $2.7\ \mu$  and it was found that the maximum value of the molecular extinction coefficient is only about 3 as compared with 35 for a solution in carbon tetrachloride, 55 for liquid water, and 120 for ice. The increase of intensity of absorption on association is thus very marked.

It is well known that there is a discrepancy between the Raman frequency 3654 ( $\nu_2$ ) and the value of 3605 deduced from infra-red data; it is shown to be due to the fact that while Bonner's relationship between the fundamental and combination frequencies is a good approximation, it is not exact enough to give a reliable value for  $\nu_2$ .

Some of the more important combination bands of liquid water are considered in relation to the vapour frequencies, and a law similar to that used by Bonner for the vapour frequencies appears to hold for the liquid. In each case the frequency in the liquid is lower than in the vapour, and so the observed shift to higher frequencies with rise of temperature naturally follows.

The structure of liquid water from the point of view of its infra-red spectrum is briefly discussed.

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**The Egyptian exploration of the Red Sea.** By A. F. MOHAMED. (*Communicated by J. Proudman, F.R.S.—Received 17 August 1939.*)

An account is given of some of the more outstanding results of a preliminary Egyptian Expedition sent to the northern Red Sea in the winter of 1934–5. The important features of the hydrography of the area investigated such as the origin of bottom water in the northern Red Sea, the water movements through the Strait of Tiran, the adiabatic temperature changes at great depths in the Gulf of Aqaba, the thermal régime of the Gulf of Suez and the nature of circulation in the Red Sea are described. The chemical observations have revealed the presence of a maximum phosphate layer at intermediate depths in the Red Sea; but at these depths the oxygen concentration as well as the pH is at its minimum. The analysis data of thirty-eight bottom samples collected from the northern Red Sea and its adjoining gulfs suggest that the environment conditions of sedimentation are different in each of the three main regions investigated.

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**Critical and co-operative phenomena. V. Specific heats of solids and liquids.** By A. F. DEVONSHIRE. (*Communicated by J. E. Lennard-Jones, F.R.S.—Received 18 August 1939.*)

Specific heats of simple substances in the liquid or solid phase have been calculated in terms of the intermolecular forces. Comparison is made with the experimental values for krypton and argon, and fair agreement is obtained. Calculations are also made of thermal expansion and compressibility.

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**The origin of the larval kidneys of the Stylommatophora.** By W. FERNANDO. (*Communicated by E. W. MacBride, F.R.S.—Received 22 August 1939.*)

The mesodermal bands are developed from the mother cell of the mesoderm. From the mesodermal bands differentiate the rudiments of the larval kidneys in *Achatina*, *Glossula* and *Opeas*.

In *Achatina* this mesodermal rudiment isolates from the remainder of the mesoderm and develops a lumen inside. An ectodermal invagination later unites with the mesoderm and a tubular larval kidney results, opening at one end to the exterior. In *Glossula* the isolation of the mesodermal rudiment is less marked than in *Achatina*, but subsequent development is the same. In *Opeas* the mesodermal rudiment of the larval kidney is differentiated at a very early stage while still in contact with the rest of the mesodermal band. The ectodermal invagination also develops very early but the union of the two portions takes place at a later stage.

The larval kidneys of the Stylommatophora are for the greater part mesodermal, only a small portion with the external aperture being formed of ectoderm; the larval mesoderm take no part in their formation.

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**Some cases of the steady two-dimensional percolation of water through ground.** By B. DAVISON and L. ROSENHEAD. (*Communicated by J. Proudman, F.R.S.—Received 22 August 1939.*)

The paper contains a summary of the mathematical theory of two-dimensional ground-water motion. It also contains a lemma which in special cases simplifies the solution. As examples involving the application of the lemma three cases are considered. They are:

(i) The characteristics of ground-water motion in a broad embankment with vertical faces, due allowance being made for evaporation from the free surface or water table. In this problem formulae are established for the volumes of fluid entering and leaving the embankment in unit time when there is no seepage surface and when evaporation takes place over the free surface. A condition is deduced upon which the existence of the seepage surface depends.

(ii) The characteristics of ground-water motion in the soil outside an irrigation dyke of rectangular section, the base being horizontal and at a constant height above a plane impervious surface. Evaporation is taken into account.

Formulae are obtained, expressed in terms of integrals, for the quantity of fluid entering the soil in unit time and for the width, at the impervious surface, of the strip of soil irrigated by the dyke.

(iii) The characteristics of ground-water motion in the soil outside a number of parallel draining tubes. The equation of the free surface is obtained.

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**The emission spectrum of hydrocarbon flames.** By E. C. W. SMITH. (*Communicated by A. C. G. Egerton, Sec.R.S.—Received 23 August 1939.*)

An examination has been made of the emission of stationary and explosion flames of ethylene in air, enriched air and oxygen, and of the explosion flames of propane in air, together with a chemical examination of the condensable products of reaction. The explosive limits of these mixtures have also been determined.

It is suggested that the carbon deposition observed at pressures in excess of some critical value with ethylene mixtures may be due to the polymerization of  $C_2$  molecules. It would appear that the "green flame" regions observed by Townend and Chamberlain and by Townend and Hsieh for various ethers and hydrocarbons, are determined solely by the stability of the same species.

It is suggested that the so-called "ethylene flame bands" which also occur in the spectra of propane and other hydrocarbon flames are possibly emitted by an isomeric formaldehyde molecule,  $HC.OH$ .

**Determinations of the Rydberg constants,  $e/m$  and the fine structure of  $H_\alpha$  and  $D_\alpha$  by means of a reflexion echelon.** By J. W. DRINKWATER, SIR OWEN RICHARDSON, F.R.S., and W. E. WILLIAMS. (*Received 23 August 1939.*)

The wave numbers of the main lines of  $H_\alpha$  and  $D_\alpha$  have been directly determined *in vacuo* by means of a 40-plate reflexion echelon. The liquid air cooled discharge tube containing helium with a small percentage of hydrogen (or deuterium) gave approximately the theoretical ratio between the intensities of the "apparent doublets" over the optimum range of operating conditions, which was investigated. With 6440.2491 Å as the vacuum wavelength of the red cadmium line, the wave number of the "centre of intensity" of the main line of  $H_\alpha$  is  $15,233,067_0 \text{ cm.}^{-1}$  (r.m.s. error  $0.001_4 \text{ cm.}^{-1}$ ) and the Rydberg constant  $R_H = 109,677.58_3 \text{ cm.}^{-1}$  (r.m.s. error  $0.01_5 \text{ cm.}^{-1}$ ). For the heavier isotope the corresponding values are  $15,237,211_2 \text{ cm.}^{-1}$  (r.m.s. error  $0.001_3 \text{ cm.}^{-1}$ ) and  $R_D = 109,707.42_1 \text{ cm.}^{-1}$ .

The mass of the electron, using the atomic values of Bainbridge is

$$m = (5.48646 \pm 0.0005) \times 10^{-4} \text{ atomic units.}$$

Taking the Faraday as  $9651.31 \pm 0.80$  coulombs we get

$$e/m = (1.7591 \pm 0.0004) \times 10^7 \text{ e.m.u./g.}$$

With  $e/M_H = 9573.48$  we obtain  $M_H/m = 1837.4_6$  and  $R_\infty = 109,737.27_8 \text{ cm.}^{-1}$ .

Seven microphotometer records of  $D_\alpha$  and four of  $H_\alpha$  have been analysed; denoting the components in order of their relative intensities and  $\hat{14}$  to indicate the centre line of the unresolved doublet of the first and fourth component (termed the main line) we find  $\hat{14}-2$  for  $D_\alpha = 0.320_0 \text{ cm.}^{-1}$  with a spread of  $0.004 \text{ cm.}^{-1}$  and  $2-3 = 0.119 \text{ cm.}^{-1}$  with a spread of about twice the amount. The observed half-width values for the second and third components are  $0.094$  and  $0.108 \text{ cm.}^{-1}$ , the latter being frequently obviously unsymmetrical. An analysis of the  $\hat{14}$  doublet places component 4 at  $0.04_4 \text{ cm.}^{-1}$  from component 1. The corresponding values for  $H_\alpha$  are  $0.319_6$  and  $0.131_1 \text{ cm.}^{-1}$  with half-widths of  $0.130$  and  $0.136 \text{ cm.}^{-1}$ .

Although these values appear to support Pasternack's suggestion of a perturbation



of the  $2^{\text{nd}}$  level, the increased intensity of the third component coupled with an increased half-width, in addition to other evidence, gives us ground for supposing that the discrepancies are caused by molecular lines in this region. We conclude that no real evidence has yet been obtained to show that the fine structures depart substantially from the values calculated from Dirac's equations.

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**The pigmentary effector system, IX. The receptor fields of the teleostean visual response.** By L. HOGGEN, F.R.S., and F. LANDGREBE. (*Received 24 August 1939.*)

Chromatic behaviour of *Gasterosteus* is controlled by a humoral and by a nervous mechanism. The former alone plays any significant part in the transition to the equilibrium condition in darkness. It reinforces nervous control in the transition to the equilibrium condition during background reversal with overhead illumination.

The photoreceptors concerned with the black background response are located in the floor of the retina below the optic nerve, and the photoreceptors concerned with the white background response are located in a restricted region in the centre of the retina above and below the optic nerve. With respect to colour change the dorsal region of the retina is neutral.

The lens of the eye of *Gasterosteus* consists of an outer spherical shell and a concentric spherical core. The refractive index of the former, like that of the vitreous humour, does not differ significantly from that of water. The core which has a high refractive index in the neighbourhood of 1.5 is the effective refractive constituent of the optical system.

In relation to image formation, we may distinguish three regions of the retina: (a) below the optic nerve, a small area where images of extra-aqueous objects are focussed; (b) around the optic nerve, a larger region where images of sub-aqueous objects are formed; (c) a band of the periphery where only light and shadow are registered.

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**The reactions of the urinary bladder of the cat under conditions of constant volume.** By the late J. MELLANBY, F.R.S., and C. L. G. PRATT. (*Received 28 August 1939.*)

Instantaneous change from constant pressure to constant volume conditions produces either an isometric contraction or a state of quiescence at zero pressure, according to the phase of the isotonic rhythm at which the change is made. The isometric contraction may be followed by a state of quiescence at zero pressure for an indefinite period, or by a rhythmic series of similar contractions. The bladder nearly always relaxes to zero pressure in the intervals between isometric contractions. The isometric contraction differs from an isotonic rhythmic contraction in several particulars, which are described. The hydrostatic pressure reached during an isometric contraction may be more than 100 mm. Hg. The isometric contraction is probably reflex in nature.

Under light chloralose anaesthesia, and in the decerebrate cat, the height of the isometric contraction may be accompanied by twitches of the diaphragm, abdominal

muscles and limb muscles. Division of the hypogastric nerves does not modify the isometric contraction. Stimulation of the peripheral end of the cut hypogastric nerves causes a submaximal isometric contraction, followed by a period of diminished excitability. Division of the nervi erigentes abolishes the isometric contraction. Stimulation of the peripheral ends of the cut nervi erigentes causes a maximal isometric contraction.

Adrenaline produces contraction, after a long latent period, similar to the isometric contraction. Acetylcholine produces a prompt contraction, similar to the isometric contraction. Atropine abolishes the isometric contraction more readily than it destroys the isotonic rhythm.

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**Calcium and blood coagulation.** By the late J. MELLANBY, F.R.S., and C. L. G. PRATT. (*Received 25 August 1939.*)

The calcium content of fowl serum is the same as that of plasma. Therefore calcium does not combine with fibrinogen in the formation of fibrin.

Calcium has no influence on the rate of coagulation of plasma by thrombase. Precipitation of calcium from fowl plasma shows that only 8-9 mg. % may be readily precipitated by a soluble oxalate. A great excess of oxalate is required to precipitate the remaining calcium. The coagulation of fowl plasma by thrombokinase is prevented when the calcium readily precipitated by oxalate is removed. A concentration of calcium ion of less than 0.3 mg. % is required for the coagulation of fowl plasma by thrombokinase.

The rate of activation of prothrombase by thrombokinase is accelerated by the presence of calcium ions. The spontaneous activation of prothrombase in the presence of water, acetic acid and oxalic acid, shows that the calcium ion increases the rate of the reaction, but is not essential to the reaction.

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**Studies in peroxidase action. II. The oxidation of *p*-toluidene.** By P. J. G. MANN and B. C. SAUNDERS. (*Communicated by Sir William Pope, F.R.S.—Received 25 August 1939.*)

The enzyme peroxidase derived either from horseradish or turnips readily oxidises *p*-toluidine, at room temperature, in the presence of dilute hydrogen peroxide at pH 4.5 (dilute acetic acid solution).

A red coloration is at first produced and then a red-brown solid gradually separates. This has been shown to consist of 5-amino-toluquinone-bis-*p*-tolylimine, 5-*p*-toluidino-toluquinone-bis-*p*-tolylimine, 4:4'-dimethyldiphenylamine, a small quantity of 4:4'-dimethylazobenzene and very small quantities of 5-amino-toluquinone-*p*-tolylimine, 5-*p*-toluidino-toluquinone-*p*-tolylimine and of a substance of m.p. 167°. The action of hydrogen peroxide and ferrous sulphate (in place of the enzyme) on *p*-toluidine dissolved in dilute acetic acid is different from the reaction described above.

A convenient form of apparatus for the continuous elution of a chromatogram is described.

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**Resonance phenomena in the disintegration of fluorine by protons.** By W. E. BURCHAM and S. DEVONS. (*Communicated by J. D. Cockcroft, F.R.S.—Received 25 August 1939.*)

An investigation has been made of the short range  $\alpha$ -particles emitted from fluorine under proton bombardment. The  $\alpha$ -particles were separated from the scattered protons by magnetic deflection. The results confirm the view that the resonant  $\gamma$ -radiation is emitted from an excited level of  $^{18}\text{O}$  formed after emission of a low energy  $\alpha$ -particle. A description is given of some measurements of the widths of the resonances for  $\gamma$ -radiation, using magnetically resolved beams of protons of high homogeneity. The results indicate that the resonances are very narrow in two cases examined, and considerably broader in the third. The excitation function for the emission of 5.9 cm.  $\alpha$ -particles in the reaction  $^{19}\text{F} + ^1\text{H} \rightarrow ^{18}\text{O} + ^4\text{He}$  has been examined carefully. The excitation function shows resonant features superposed upon a background which increases with increasing proton energy. A discussion of some possible explanations of the resonant phenomena observed is given.

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**Experiments on the transmutation of sodium by deuterons.** By E. B. M. MURRELL and C. L. SMITH. (*Communicated by J. D. Cockcroft, F.R.S.—Received 25 August 1939.*)

The transmutations of sodium under bombardment with deuterons of energy  $0.85 \times 10^6$  eV have been investigated in detail.

The two groups of  $\alpha$ -particles which are emitted, with ranges  $5.0 \pm 0.1$  and  $3.4$  cm., are attributed to the process



The energy of the longer range group leads to a value for the mass of  $^{23}\text{Na}$  of  $22.9961 \pm 0.0003$  and the shorter range group indicates the presence of an excitation level in the  $^{21}\text{Ne}$  nucleus which is calculated to be  $1.6 \times 10^6$  eV above the ground state.

Four groups of protons were also found to be emitted with ranges of 7.5, 24.0, 33.4 and 37.8 cm. From the energy of the group of longest range a value of  $23.9976 \pm 0.0003$  is deduced for the mass of the  $^{24}\text{Na}$  atom. From the energy values of the other groups it is concluded that excitation levels at 0.38, 1.26 and  $3.38 \times 10^6$  eV above the ground state exist in the  $^{24}\text{Na}$  nucleus.

The value  $23.9913 \pm 0.0003$  is also deduced for the mass of  $^{24}\text{Mg}$ .

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**The specific heat of superconducting mercury, indium and thallium.** By A. D. MISENER. (*Communicated by J. D. Cockcroft, F.R.S.—Received 25 August 1939.*)

Threshold field curves for mercury, indium and thallium were accurately determined by a magnetic method down to a temperature of  $1.1^\circ\text{K}$ . By least-square methods, polynomials were fitted to these curves and from these the specific heat of the metals in the superconducting state was calculated using the formulae of Gorter

and Casimir. The values obtained for thallium agree with the direct determinations of Keesom and Kok. The specific heat of superconducting mercury and thallium varies as  $T^3$ , that of indium, as  $T^4$ .

**The thermal capacity of pure iron.** By J. H. AWBERY and E. GRIFFITHS, F.R.S. (*Received 25 August 1939.*)

The specific heat of very pure iron has been measured by an electrical adiabatic method up to nearly  $1000^\circ\text{C}$ . A sample weighing about 1200 g. was heated by an internal heater, and a subsidiary furnace was used to maintain a jacket around the specimen at the same temperature as the latter throughout the experiment. There was thus no net loss of heat from the specimen and the specific heat was given directly by measuring the energy input to the internal heater and the corresponding temperature rise. The method was a continuous one, i.e. the internal heater was in action throughout a long period, the times at which the specimen passed each of a number of temperatures being noted, so as to obtain a number of points on the specific-heat curve.

The specific heat rises gradually from  $0.113\text{ cal./g./}1^\circ\text{C}$  at  $50^\circ\text{C}$  to a maximum of  $0.28$  at the magnetic point,  $755^\circ\text{C}$ ; it then falls again, and subsequently rises as the transformation point at  $903^\circ\text{C}$  is approached. This "point" is found to be double. At  $903^\circ\text{C}$  the specific heat is infinite, but after falling to  $0.37$  it rises again at about  $908^\circ\text{C}$  before finally falling to the value  $0.13\text{--}0.14\text{ cal./}1^\circ\text{C}$ , which is the value found for the high-temperature modification (gamma iron). The heat absorbed at the transformation is  $3.9\text{ cal./g.}$

**On the production of Ra E and polonium by deuteron bombardment of bismuth.** By D. G. HURST, R. LATHAM and W. B. LEWIS. (*Communicated by J. D. Cockcroft, F.R.S.—Received 28 August 1939.*)

The Cambridge cyclotron has been used to examine the reactions occurring when bismuth is bombarded with deuterons of energies up to  $8.7\text{ MeV}$ .

The reactions found to take place are the formation of Ra E by a  $(d, p)$  process, and of Po by a  $(d, n)$  process. The Ra E subsequently decays into Po by the emission of an electron; with a 5 days' half-life.

By determining the amounts of Po formed directly, and indirectly, following the decay of Ra E, it has been possible to measure the relative yields of the  $(d, p)$  and  $(d, n)$  reactions at various energies of the deuteron.

The excitation functions for the two reactions have been compared with the values to be expected on theoretical grounds.

**The equation  $d^2 \log y/dx^2 = 1 - y$  when  $y > 0$ .** By J. CARSON and L. F. RICHARDSON, F.R.S. (*Received 29 August 1939.*)

The equation  $d^2 \log y/dx^2 = 1 - y$  defines a manifold of periodic functions. Certain psychological and physiological oscillations are represented approximately by

particular integrals. The equation arose in connexion with the relaxation oscillations of a neon lamp. The manifold of functions has two parameters, which may be taken to be the co-ordinates  $x = x_0$ ,  $y = G$  of a particular maximum of  $y$ . Alteration of  $x_0$  merely moves the wave as a rigid shape parallel to the  $x$ -axis, so that for many purposes we may take  $x_0 = 0$  and regard  $G$  as the interesting parameter of the family. Alteration of  $G$  alters both the wave-form and the wave-length  $\lambda$ .

$$\text{As } G \rightarrow 1, \lambda \rightarrow 2\pi\{1 + \frac{1}{24}(G-1)^2 \dots\}. \quad \text{As } G \rightarrow \infty, \lambda \rightarrow \infty.$$

At  $G = 50$ ,  $\lambda = 20.28$ . The wave-form for  $G = 100$ , consists of narrow steep peaks separated by broad flat-bottomed valleys; the minimum value of  $y$  being  $3.8 \times 10^{-42}$ . For smaller values of  $G$  the peaks are less narrow and the valleys not so flat, until as  $G \rightarrow 1$  the wave-form tends to  $y = 1 + \epsilon \cos x$  where  $\epsilon \rightarrow 0$ . In order to evaluate  $y(x)$  numerical quadrature has been used. A few numerical tables are given, also a list of related problems.

This family and its connexions seem to form a coterie detached from the common tabulated functions.

**The excitation of inner electrons in zinc, cadmium and mercury by electron impact.** By A. H. LEE. (*Communicated by R. Whiddington, F.R.S.—Received 30 August 1939.*)

Certain analogous ultra-ionization potentials have been observed in zinc, cadmium and mercury, while studying the energy losses of electrons in the vapours of these elements. In order to explain these U.I.P., all the processes which could give rise to them are examined in detail. It appears that none of the processes which were formerly found to be responsible for such effects, is adequate in the present case. A new process—excitation of an inner electron—hitherto unrecognized in electron impact experiments, is shown to be the cause. The analogous U.I.P. correspond to analogous transitions observed by Beutler in the ultra-violet absorption spectra of the three elements, and attributed by him to excitation of a  $d$  electron in the closed shell immediately below the valence shell, resulting in a  $^3P_1$  state, in each case. (The valence electrons remain in their normal states.) A further U.I.P. in mercury is explained in a similar way.

The observed U.I.P. are abnormally intense for  $^1S_0$ – $^3P_1$  excitations at the bombarding voltages used. This anomaly may be a consequence of the unusual process involved. Hence an approximate determination of the excitation function of the first “inner” excitation in mercury has been made, and certain features noted.

**An investigation of cold-worked polycrystalline alpha-iron.** By L. MULLINS and J. W. RODGERS. (*Communicated by Sir Harold Carpenter, F.R.S.—Received 1 September 1939.*)

A new empirical method of approach to the mechanism of grain breakdown in metals on cold-working is described and applied in an X-ray examination of alpha-iron, cold-worked by elongation.

The individual reflections from the grains of the metal are microphotometered along the periphery of the reflection rings and these photometer records are analysed into the components which arise from the discrete crystal fragments of the deformed grain, a Gaussian error curve being the basis of the unit reflection curves used in the analysis.

A survey of the many photometer records analysed showed them broadly divisible into four types, corresponding to the reflections from the various types of actual disintegration of the metallic grain, representative curves being given.

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**Thermal transpiration of a dissociating gas and the modified dissociation formula.** By B. N. SRIVASTAVA. (*Communicated by M. N. Saha, F.R.S.—Received 5 September 1939.*)

In this paper the thermal transpiration of a dissociating gas is investigated theoretically for two chambers maintained at different temperatures and communicating with each other through a narrow opening. It is shown that the condition of thermodynamical equilibrium and the usual transpiration relation for each constituent cannot both be satisfied simultaneously. An approximate solution of the problem is given which is based on the assumption of thermodynamical equilibrium in each chamber. Expressions are deduced for the absolute magnitude as well as the ratio of the atomic or molecular concentrations in the two chambers in the general case and some limiting cases.

The problem is treated rigorously from the view-point of a steady state. Expressions are worked out showing how the law of mass action suffers modification in this case. Expressions are also deduced for the atomic and molecular concentrations in the two chambers and the modified transpiration relation is stated. Finally the relative merits and demerits of both the treatments are clearly set forth.

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**A spectroscopic investigation of hydrocarbon flames.** By W. M. VAIDYA. (*Communicated by M. N. Saha, F.R.S.—Received 5 September 1939.*)

The present paper forms part I of a series of three papers, describing the results obtained in an extensive investigation of the spectra of the flames of carbon compounds, which was undertaken in order to understand the processes of combustion in hydrocarbon flames. The flames of compounds, found by chemists to occur as intermediate products in the combustion of main hydrocarbons have also been included as also some other flames for comparative purposes. A Smithells flame-separator has been used in many cases, so as to facilitate independent observations of the inner and outer cones.

It has been found that the outer cones of common gas, methane, ethylene, acetylene, ether and ethyl alcohol give a spectrum identical with the CO flame spectrum. The inner cones of all the compounds except formaldehyde and methyl alcohol give in general  $C_2$ ,  $CH$ ,  $HO$  and the ethylene flame bands with varying intensities. Formaldehyde yields only CO flame spectrum while in methyl alcohol are present  $CH$ ,  $HO$ , and the CO flame bands.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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**Effusion phenomena in relativistic quantum statistics.** By D. V. GOGATE.  
(Communicated by Sir Owen Richardson, F.R.S.—Received 8 September 1939.)

This paper deals with the phenomenon of the effusion of energy of an ideal gas through a narrow orifice taking account of relativistic quantum statistics. In § 1 non-degenerate matter is considered and the amount of energy effusing out per second per unit area is calculated. In § 2 the effusion of energy is calculated for a completely degenerate gas, degenerate in the sense of Fermi-Dirac statistics. It is also shown that in the case of complete degeneracy, the same expression holds for the rate of effusion of mass throughout the whole region from completely non-relativistic to completely relativistic. The results are displayed graphically in § 3 to bring out clearly the physical significance of the different formulae for the various cases of non-degeneracy and degeneracy.

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**Relaxation methods applied to engineering problems. V. Conformal transformation of a region in plane space.** By R. W. G. GANDY and R. V. SOUTHWELL, F.R.S. (Received 14 September 1939.)

Part III of this series dealt (*inter alia*) with the application of relaxation methods to problems in plane-potential theory. In this paper the problem of conformal transformation is discussed as a particular example.

Orthodox mathematics presents the transformation in an equation of the type

$$\alpha + i\beta = f(x + iy),$$

which expresses a one-to-one relation between points in the original and in the transformed region; but the equation is of no concern to the practical computer provided that he can construct "maps" of which it is the mathematical expression, and for this it is only necessary to have  $\alpha$  and  $\beta$  evaluated at nodal points at some regular "net". Thus the problem from a practical standpoint is to construct the  $\alpha$ - $\beta$  contours. In this paper four cases of common occurrence are treated, and the " $\alpha$ - $\beta$  maps" are reproduced. The accuracy of the calculations in general is greater than a drawing can exhibit.

Some incidental problems call for notice which were not confronted in Part III. Thus whereas one of the two conjugate functions  $\alpha$ ,  $\beta$  can be computed by the methods given previously, its computed values (being only an approximation to a plane-harmonic function) are not in general compatible with a single-valued conjugate. In case (c) we are concerned with a region of infinite extent, and the device of "geometrical inversion" must be applied in order to render the problem tractable by relaxation methods. For some applications we require values of the ratio

$$h = |d(\alpha + i\beta)/d(x + iy)|$$

at least as accurate as those of  $\alpha$  or  $\beta$ : examples are given to show that the requisite accuracy is attainable, use being made (when necessary) of the fact that  $\log h$  is plane-harmonic.

The last problem treated (calculation of the electric capacity of a straight cable or condenser) is in essence an example of conformal transformation but can be solved when only one of  $\alpha$ ,  $\beta$  has been computed. It does not demand construction of the  $\alpha$ - $\beta$  map.

**Structure and thermal properties associated with some hydrogen bonds in crystals. III. Further examples of the isotope effect.** By A. R. UBBELOHDE. (*Communicated by Sir William Bragg, P.R.S.—Received 20 September 1939.*)

The effect on the lattice spacings of substituting deuterium for hydrogen has been investigated for pentaerythritol,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ , fumaric acid,  $\text{KH}_2\text{PO}_4$ ,  $\text{NaHSO}_4$ , urea and  $\text{KHF}_2$ . The main conclusion is that when the crystal structure contains hydroxyl bonds the isotope effect is small, and when it contains hydrogen bonds there is a marked expansion, which disturbs the lattice sufficiently to lead to the separation of potassium phosphate in a new crystal structure. The effect for potassium hydrogen fluoride does not suggest a short hydrogen bond in this compound. Short hydrogen bonds seem to require special resonance forces for their explanation.

**The phosphorolysis of starch by an enzyme system from pea seeds.** By CHARLES S. HANES. (*Communicated by F. F. Blackman, F.R.S.—Received 2 September 1939.*)

An investigation of a system of enzymes from pea seeds which catalyses the formation of hexose-phosphates from starch, various dextrans, and maltose is described.



The first recognizable step in the process is the formation of glucose-1-phosphate which has been isolated in the form of the crystalline potassium salt. Evidence is advanced supporting the conception that this ester is formed as the result of a process of direct phosphorolytic cleavage of terminal glucose units from the non-aldehydic ends of the chain molecules of the substrate.

This primary conversion of starch into glucose-1-phosphate is shown to be a reversible reaction, catalysed in both directions by an enzyme which has been termed phosphorylase.

Glucose-1-phosphate takes part in an alternative reaction, catalysed by the phosphoglucose conversion system, in which it is converted into reducing hexose-monophosphates.

Finally, in non-dialysed extracts, fructose-diphosphate is formed.

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**The chromatic behaviour of the eel.** By H. WARING. (*Communicated by L. Hogben, F.R.S.—Received 14 September 1939.*)

There is a dominant bi-humoral control of the dermal melanophores of the eel. The expanding hormone "B" is localized in the posterior lobe of the pituitary; the contracting hormone "W" is localized in the anterior half of the pituitary. "B" is built up quickly and excreted quickly; "W" is built up slowly and excreted slowly.

There is direct innervation of the melanophores. The time relations of the natural responses show that it has no significance in the behaviour of normal animals. It can only be distinguished in the absence of "B". Release from nervous control might be expected to show itself in the transition from a white illuminated ground to darkness, but the normal behaviour is dominated by the slow excretion of "W".

The results of this investigation support the general hypothesis of chromatic co-ordination outlined by Hogben and Landgrebe.

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**The fine structure of phloem fibres in untreated and treated hemp.** By B. C. KUNDU and R. D. PRESTON. (*Communicated by V. H. Blackman, F.R.S.—Received 21 September 1939.*)

During recent years many investigations of wall structure have been based on the use of swelling agents. While it is not to be denied that observations of swollen material are useful in some respects, it is the aim of the present paper to show that extreme care must be taken in the deduction from them of the nature of the intact wall. Several methods lead to the conclusion that in the hemp fibre the wall is composed of cellulose chains running almost longitudinally; even the tenuous outer layers do not deviate considerably from this orientation. On the other hand, the direction of cellulose chains in the swollen walls depends on a number of factors, including the nature of the swelling treatment, the distribution in the wall of the "incrusting substances", and the chain direction in the wall before swelling. It appears to be a general rule that the greater the swelling, the more inclined to the

longitudinal the cellulose chains become, and this effect becomes the more marked as the material is less carefully manipulated. The presence of an internal layer, with slight response to swelling agents, leads to the appearance of internal fibrils which retain their longitudinal orientation. This combination, in one swollen wall, of cellulose chains running in different directions, which is clearly an artefact due to swelling, is responsible for the erroneous conception of the intact wall as built on similar lines. It seems certain that this effect of swelling may be even more marked with other types of cell.

The phenomenon of "ballooning" is to be explained in terms of the chemical nature of the various wall layers; there is no need to invoke the presence in an outer layer of cellulose chains oriented transversely. The outer highly lignified layer is much more resistant to swelling than is the central layer and, upon rapid swelling of this latter layer, becomes fractured along a steep spiral line, coincident with the direction of the cellulose chains. Further swelling causes this spiral to be flattened until it assumes the form of transversely oriented fibrils. This appearance again is clearly an artefact. A phenomenon resembling the well-known "chemical sectioning" also receives explanation on similar lines. Resistance to swelling may be due either to high lignin content (outer wall layer) or to the precise configuration of the cellulose itself (inner wall layer).

In the interpretation of swelling phenomena, the greatest care must be taken in observing the changes which occur during the swelling process. In hemp there is nowhere the slightest evidence that in any layer the cellulose chains run in a direction very different from those composing the bulk of the wall.

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**The lattice spacings of the primary solid solutions of silver, cadmium and indium in magnesium.** By G. V. RAYNOR. (*Communicated by W. Hume-Rothery, F.R.S.—Received 27 September 1939.*)

Accurate measurements have been made of the lattice spacings of the primary solid solutions of silver, cadmium and indium in magnesium. At equiatomic compositions, decreasing the valency of the solute increases the lattice distortion in the basal plane. In dilute solution, indium, cadmium and silver contract the "a" parameter by amounts proportional to 1 : 2 : 5 respectively, while the volume of the unit cell is contracted by amounts proportional to 1 : 3 : 9 respectively. The "a" parameter factors may be expressed as  $1 + (3 - \text{valency})^2$  and the volume factors as  $1 + 2(3 - \text{valency})^2$ . There is no such regularity for the "c" parameters. The results may be interpreted qualitatively in terms of the Brillouin zone theory of metals.

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**The molecular spectrum of iodine excited by fluorescence in the presence of nitrogen and by active nitrogen.** By A. ELLIOTT. (*Communicated by S. R. Milner, F.R.S.—Received 28 September 1939.*)

The fluorescence spectrum of iodine vapour in the presence of nitrogen at 1 atm. pressure has been photographed. The spectrum contains four separate band systems. A vibrational analysis has been carried out for two of these systems, one of which is

often referred to as the "continuum" 3425, and which is due to a  $^1\Sigma_u^+ \rightarrow ^1\Sigma_g^+$  transition. The effect of varying nitrogen pressures on the intensities of the bands has been examined.

The spectrum of iodine vapour excited by active nitrogen has been photographed and compared with the fluorescence spectra of iodine vapour in the presence of nitrogen.

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**The properties of paramagnetic salts at low temperature.** By J. SAUER and H. N. V. TEMPERLEY. (*Communicated by R. H. Fowler, F.R.S.—Received 29 September 1939.*)

An attempt is made to examine theoretically the properties of paramagnetic salts at very low temperatures. The model taken is a lattice of freely suspended small magnets, but mathematical difficulties prevent a rigorous calculation of the partition function, either on classical or quantum lines. A simple model is proposed, which is really a generalization of the Bragg-Williams theory, enabling one to take account of the effect of a magnetic field. The few configurations whose energies are known are used for fixing arbitrary constants in the general expression for the energy. The theory predicts that the state of lowest energy is either a spontaneously magnetized state or a state in which alternate rows of magnets point in opposite directions, according to the shape of the specimen. A sphere should not be spontaneously magnetized, but will be in an anti-parallel state at low temperatures, but spontaneous magnetization should appear in an ellipsoid with an eccentricity greater than a certain critical value. The transition curve bounding the region in which the anti-parallel state is stable consists partly of a line of Curie points, which corresponds to the horizontal line in the approximate diagram and partly consists of a line of points determining transitions of the first order, with a definite latent heat. This line is represented by a vertical line in the approximate diagram. The effect of shape on the properties of the specimen seems to be established experimentally, but it is difficult to make numerical predictions, owing to the rough nature of the theory.

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**Seismic investigations on the Palaeozoic floor of East England.** By E. C. BULLARD, T. F. GASKELL, W. B. HARLAND and C. KERR-GRANT. (*Communicated by Sir Gerald Lenox-Conyngham, F.R.S.—Received 29 September 1939.*)

The depth of the Palaeozoic floor under part of East England has been investigated by the refraction seismic method. Records have been taken every 200 ft. along lines 4000–8000 ft. long; such detailed shooting enables various sources of uncertainty in the results to be investigated.

The interpretation of the seismic results required a more thorough knowledge of the contours of the Jurassic and Cretaceous than was available; the data from bore holes and outcrops have therefore been collected and are presented in the form of

contoured maps showing the depths of various horizons and the thicknesses of rock between them.

A map of the form of the Palaeozoic floor and a discussion of its constitution are also given. The latter is based on a re-examination of the bore hole cores and on the seismic velocities.

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**The steady two-dimensional flow of viscous fluid between plane walls.**  
By L. ROSENHEAD. (*Communicated by G. I. Taylor, F.R.S.—Received 2 October 1939.*)

This paper considers the steady radial two-dimensional flow of viscous fluid between plane walls which either converge or diverge. A general solution is obtained in terms of elliptic functions and the various mathematically possible types of flow are discussed. The problem is treated numerically and  $\alpha$ , the semi-vertical angle between the walls, is plotted against  $R$ , the Reynolds number, for the various types of flow.

The investigations show that for every pair of values of  $\alpha$  and  $R$  the number of mathematically possible velocity profiles is infinite. The effect of increasing the Reynolds number in outflow is to exclude, progressively, more and more of the simpler types of flow. No such exclusion is introduced when  $R$  is increased in inflow. It is shown also that when  $\pi > \alpha > \frac{1}{2}\pi$  pure outflow is impossible. The fluid must contain regions of outflow and inflow. Further, when  $\pi > \alpha > \frac{1}{2}\pi$  there is a range of values of small Reynolds number in which pure inflow is impossible. With increasing  $R$  in pure inflow, and with small values of  $\alpha$ , the velocity profile exhibits all the well-known characteristics of boundary layers near the walls and an approximately constant velocity across the rest of the channel. In pure outflow the flow becomes more and more concentrated in the centre of the channel as  $R$  is increased until finally regions of inflow occur near the walls.

Speculative assumptions are introduced to suggest a plausible sequence of changes of the velocity profile as the Reynolds number is increased.

The paper does not investigate the stability of the various types of flow.

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**The effect of adaptation on subjective brightness.** By K. J. W. CRAIK.  
(*Communicated by F. C. Bartlett, F.R.S.—Received 3 October 1939.*)

Subjective brightness has been investigated by binocular matching. The adapted response "saturates" at 1000 e.f.c.; below this it decreases less rapidly than initial brightness in the proportion, roughly, of 1:2 on a logarithmic scale of stimulus intensities. Adaptation thus compensates subjective brightness for slow changes in illumination, perfectly above 1000 e.f.c. and less perfectly below this. The adapted level of subjective brightness is relatively low; e.g. the initial brightness of 3 e.f.c. exposed to the dark adapted eye equals that of 15,000 e.f.c. to an eye adapted to that illumination.

The data on subjective brightness fit well into a photochemical theory of vision. The difficulties of accounting similarly for some of the features of brightness discrimination under a fixed adapting illumination are discussed, and it is suggested

that effects due to the neural system are superimposed on those due to the photochemical system, and that this neural system involves nerve-endings of different types whose behaviour under a range of illuminations, denoted as the lower non-linear, linear, and upper non-linear or overload ranges, is better described in terms of their own excitability, adaptive and refractory-period characteristics than in terms of the underlying photochemical mechanism.

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**The photodynamic activity of the tissues of mice treated with 3 : 4 benzpyrene.** By J. C. MOTTRAM. (*Communicated by J. W. Cook, F.R.S.—Received 6 October 1939.*)

The photodynamic action of 3 : 4-benzpyrene on *Paramoecium* occurs in dilutions of the order of one part in a hundred million. By this means its presence has been looked for in the tissues of mice either painted or inoculated with this hydrocarbon. Apart from the site of its application it was found to be present only in the liver and the lungs. It is noteworthy that these are the only two sites apart from its place of application where tumours have been observed to occur in mice treated with carcinogenic agents. It appears therefore that the reason why tumours occur in the liver and the lungs is because the hydrocarbon accumulates in these two tissues.

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**The nature of the oestrogenic substances produced during the demethylation of anethole.** By N. R. CAMPBELL, E. C. DODDS and W. LAWSON. (*Communicated by Sir Robert Robinson, F.R.S.—Received 6 October 1939.*)

Demethylation of anethole by heating with potassium hydroxide and alcohol under pressure produces three oestrogenic substances of which one, produced in very small amount, is intensely active. These three have been isolated and identified, the most potent being 4 : 4'-dihydroxy- $\gamma$  :  $\delta$ -diphenyl-*n*-hexane. Syntheses of these and of several other dihydro dimerides of *p*-propenyl phenol are also described.

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**The pressure-voltage characteristic of the Geiger-Muller counter.** By W. SCHAEFFER. (*Communicated by B. F. J. Schonland, F.R.S.—Received 9 October 1939.*)

An approximate quantitative theory of the starting potential of the Geiger-Muller counter is given on the assumption that the replacement electrons are set free by the photo-electric effect. The gas volume is divided into three coaxial regions; an inactive zone in which the electrons acquire too little energy from the field for excitation or ionization, an excitation zone, and a zone in which both excitation and ionization occur. The theory is applied to an experimental pressure-starting potential curve and

satisfactorily accounts for the shape of the *whole* curve. It gives an approximately correct value of the ionization potential of the gas and also gives reasonable and fairly consistent values for some other physical constants and for the ratios and products of such constants. The experimental procedure used to obtain the pressure-starting potential curve is briefly described. A discussion gives some evidence for the photo-electric theory of the discharge and shows that, while the present work is by no means certain evidence for that theory, it is sufficiently probable to merit further test.

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**A new derivation of the quadratic equation for the masses of the proton and electron.** By SIR ARTHUR EDDINGTON, F.R.S. (*Received 10 October 1939.*)

The paper develops an improvement of method which considerably simplifies the physical problems treated in *Relativity Theory of Protons and Electrons*. The main difference is that by making fuller use of the conception of *degeneracy* (arising from symmetry) the cumbersome treatment of volume-elements of phase space attached to the wave vectors is avoided. The various numerical coefficients appearing in my previous results are recognized as the weights of degenerate states. This makes them easier to handle in further developments, besides making them less mysterious from the point of view of ordinary quantum theory. Before the degeneracy method could be employed it was necessary to formulate more precisely the connexion between the relativistic description of observables as relations between an object system and a standard physical environment (the comparison fluid) and the current quantum theory which ostensibly deals with the object system alone. This occupies the earlier part of the paper.

The problem treated in detail by this method is the derivation of the equation  $10m^2 - 136mm_0 + m_0^2 = 0$  whose roots are the masses of the proton and electron.

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**The masses of the neutron and mesotron.** By SIR ARTHUR EDDINGTON, F.R.S. (*Received 10 October 1939.*)

The development of relativistic quantum theory in an accompanying paper is applied to the neutron and mesotron.

The excess of the mass of the neutron over the mass of the hydrogen atom is found to be  $1.5 m_0$ , confirming a tentative derivation by H. O. W. Richardson. The magnetic moment is also found.

The mass of the mesotron is found to be  $173.2 m_0$ . A rough indication of the life-time is given.

Certain conclusions in regard to nuclear structure are derived; in particular, mesotrons are quite distinct from Yukawa particles.

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# ABSTRACTS

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**The neutrons from the disintegration of fluorine by deuterons.** By T. W. BONNER. (*Communicated by J. D. Cockcroft, F.R.S.—Received 16 October 1939.*)

The neutrons from the disintegration of fluorine by deuterons have been investigated by the method of observing helium or hydrogen recoils in a high pressure cloud-chamber. When targets containing fluorine were bombarded with 950 kV deuterons, several groups of neutrons were observed. The disintegration  $Q$  values computed from the energies of the neutron groups are 10.80, 9.33, 6.62, 5.39, 3.53, 1.84 and 0.74 MeV. The nuclear reaction appears to be ( $F^{19}$ ,  $H^2$ ,  $Ne^{20}$ ,  $n^1$ ). The disintegration value  $Q = 10.80 \pm 0.20$  MeV corresponds to a transition to the ground state of  $Ne^{20}$  and smaller  $Q$  values indicate excited states in  $Ne^{20}$  at 1.5, 4.2, 5.4, 7.3, 9.0 and 10.1 MeV. Only a small fraction of the neutrons belong to the group of maximum energy. The excited states in  $Ne^{20}$  at 5.4, 7.3, 9.0 and 10.1 MeV are unstable against alpha-particle emission and so they may break up into  $O^{16}$  and an alpha-particle. The experimental width of the level in  $Ne^{20}$  at 10.1 MeV appears to be not greater than about 0.2 or 0.3 MeV.

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**On aspects of animal locomotion. (Croonian Lecture.)** By J. GRAY, F.R.S. (*Received 17 October 1939.*)

The available facts appear to establish five main points: (i) To a surprising degree, normal locomotion is independent of the higher nervous centres, although in vertebrates the integrity of the medulla oblongata is essential for a display of full locomotory activity. In some vertebrates the role of the medulla can be replaced by appropriate electrical stimuli applied to the nerve cord. (ii) The type of locomotion

displayed by an animal can frequently be shown to depend upon the presence of a particular pattern of exteroceptive stimulation: one pattern is essential for ambulation, another for swimming or for flight. (iii) Few if any animals display co-ordinated motion when their central nervous systems are completely isolated from those patterns of peripheral stimulation which reach them under conditions of normal progression. It is, however, possible to restrict the receipt of this stimulation to a comparatively small amount of the total musculature taking part in the process of locomotion. (iv) When a vertebrate limb responds by flexion to passive stretch, each of the other three limbs respond by movements which bear an unmistakable resemblance to normal locomotory co-ordination—although these latter limbs may be completely deafferented. Reflex activity in one limb thus induces a wide spread of postural pattern within the central nervous system. These phenomena are not restricted to tetrapod vertebrates: they occur in fish, and in a variety of invertebrates. (v) Strong support is available for the conclusion that peripheral reflexes play a fundamental role in normal locomotion. Against this theory are certain facts which require further consideration.

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**Statistical thermodynamics of superlattices.** By R. H. FOWLER, F.R.S. and E. A. GUGGENHEIM. (*Received 19 October 1939.*)

This paper establishes the precise statistical basis of Bethe's method of discussing order-disorder phenomena in superlattices in Bethe's first approximation. It shows that for cubic lattices of the type here considered, in which all points of one superlattice have as nearest neighbours points of the other superlattice only, Bethe's first approximation is equivalent to the use of a method which we here call the quasi-chemical method. The quasi-chemical method is an approximation which proceeds by assuming that the bond between any pair of nearest neighbours may be treated as if it were a chemical molecule, and the number of arrangements with given numbers of bonds as if the bonds did not interfere with each other.

In the simple case considered, all the details of Bethe's method and the quasi-chemical method and the approximation on which they are based, and the relationship between them, can be made perfectly explicit. There seems reason to hope that the greater power of these explicit methods may enable them to be applied successfully for more complicated lattices, to which at present only the much rougher approximation of Bragg and Williams can be carried through.

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**An experimental study of the transfer of excitation energy in solution.** By A. H. CARTER and J. WEISS. (*Communicated by G. R. Clemon, F.R.S.—Received 23 October 1939.*)

The transfer of electronic excitation energy in solution (primarily adsorbed from an external light source) has been investigated in the case of the photosensitized decomposition of oxalic acid by uranium salts. More detailed information about the mechanism of the energy transfer in this reaction has been obtained by studying it in the presence of substances ( $I^-$ ,  $Br^-$ ) which can act as acceptors for the excitation energy of the excited uranium ions.



New experimental facts are presented which cannot be explained by any of the previously advanced theories. In particular there has been observed a change in the relative amounts of the decomposition products with varying concentrations of quenching substances and hydrogen ions present in the solution. In fact the net process of the photosensitized decomposition of oxalic acid cannot be represented by the equation:  $\text{H}_2\text{C}_2\text{O}_4 = \text{CO} + \text{CO}_2 + \text{H}_2\text{O}$  as has been assumed often in the past. The experiments show the formation of formic acid and the oxidation of a certain amount of the oxalic acid to carbon dioxide, which must be taken into account. Under the most favourable conditions not more than about 50 % of the oxalic acid is decomposed into carbon monoxide (according to the above reaction), whereas up to 72 % of the oxalic acid is decomposed into formic acid under suitable conditions ( $\text{pH} \sim 5$ ).

A theory is discussed based on the non-adiabatic electron transfer process between excited uranium ions and oxalate ions. This process constitutes the elementary process of quenching of fluorescence which is identical with the primary process in the photosensitized reaction. The complex formation between the reacting ions is interpreted as a result of quantum mechanical resonance in this primary process.

In connexion with the above investigation, the quenching of the uranium fluorescence by iodide ions and the elementary processes involved have been investigated experimentally and discussed on a similar theoretical basis.

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**Theory of the vibrations of the sodium chloride lattice.** By E. W. KELLERMAN. (*Communicated by M. Born, F.R.S.—Received 26 October 1939.*)

According to Born's treatment of polar crystals the frequency equation for a vibrating crystal contains in its coefficients lattice sums which are due to long-range Coulomb forces. Using a method developed by Ewald it has been possible to find a quickly convergent form of those sums. The general formulae for the coefficients have been developed and a special application has been made to the case of sodium chloride. The coefficients and also the frequencies themselves have been calculated for forty-eight different states of vibration of the crystal which are chosen in such a way as to make possible a fair survey over the whole frequency spectrum of the crystal. It appears that the purely electrostatic derivation of the general formulae for the coefficients does not give information about the case of the residual rays. This can only be obtained by taking account of the electrodynamic boundary conditions, namely that the crystal as a whole must not emit radiation, which leads to the correct solution for the frequency of the residual rays. The formulae for the coefficients have also been used for the calculation of the elastic constants of sodium chloride.

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**The torsional flexibility of aliphatic chain molecules.** By A. MÜLLER. (*Communicated by Sir William Bragg, P.R.S.—Received 26 October 1939.*)

The dielectric polarization of two diketones is measured in a temperature range including the melting points of the substances. The material under observation consists essentially of aliphatic chain molecules carrying two dipoles. In one sub-

stance,  $C_{10}H_{10}O_2$ , these dipoles are situated on opposite sides of the chain axis and thus neutralize each other. In the other substance,  $C_{11}H_{10}O_2$ , the dipoles lie on the same side and therefore increase the dipole strength. From the polarization experiments it is concluded that in both cases considerable distortion of the chain molecules occurs in the temperature range near the melting points.

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**Cytological studies in *Oenothera* with special reference to the relation of chromosomes to nucleoli.** By P. N. BHADURI. (*Communicated by R. R. Gates, F.R.S.*—Received 27 October 1939.)

A comparative study of the relation of chromosomes to nucleoli has been made in nine species and one hybrid of *Oenothera*. The distinction between a satellited chromosome and one with secondary constriction breaks down in *Oenothera*. Both the constricted region and the appendage are well marked in most of the species. The filament, like the appendage, is Feulgen positive.

The chromosomes have been classified in some of the species according to their sizes, the situation of the primary constriction and the presence of secondary constrictions. An exact correspondence between the number of secondary constrictions and the number of nucleoli has been established in each case. This number is four in all the species except *O. angustissima* var. *quebecensis* where it is five. Determination of the position of nucleolar chromosomes in the ring is important as a means of identifying chromosomes in the two complexes of a species. The presence of four nucleoli corresponding to four secondary constrictions in (a) heterozygous species with high chromosome catenation, such as *O. Lamarckiana*, *O. Hazelae* and *O. biflora* and (b) homozygous species with seven free pairs, such as *O. Hookeri* and *O. missouriensis*, proves that the presence of four nucleolar chromosomes is an older character than chromosome linkage. This supports the view that ring formation in *Oenothera* has evolved in the genus. That morphological changes of chromosomes can be brought about by segmental interchange is evidenced by the fact that at least one heteromorphic pair of Sat-chromosomes in *O. Lamarckiana* has become homomorphic in its mutant *O. blandina*. There is variation in the sizes of the nucleoli in species and hybrids of *Oenothera*. *O. Lamarckiana* has one very small, one quite big and two intermediate nucleoli, whereas in the homozygous *O. blandina* and *O. Hookeri* there are two distinct pairs of nucleoli. An unpaired condition of nucleoli in *Oenothera* therefore indicates heterozygosity of the species. Prochromosomes, which are Feulgen positive, have been observed in somatic cells of some species. The catenation of three species and nine interspecific hybrids has been determined. In a narrow-leaved trisomic mutation of *O. Hazelae* the fifteen chromosomes showed irregularities in catenation.

The presence of four nucleoli corresponding to four secondary constrictions, the frequent presence of multivalent chromosomes in the pollen mother cells of haploid and diploid plants, as well as the fact that no common basic number is found in the family Onagraceae, indicates that the haploid number 7 in *Oenothera* is a derived number. On this assumption *Oenothera* species cannot, therefore, be looked upon as true diploids.

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# ABSTRACTS

## OF PAPERS COMMUNICATED TO THE ROYAL SOCIETY OF LONDON

In accordance with a resolution of Council, summaries or abstracts of papers are to be published as soon as practicable. The publication of such abstracts in no way indicates that the papers have been accepted for publication in any fuller form. These abstracts are issued for convenience with the "Proceedings of the Royal Society of London" but do not form a part of the "Proceedings".

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15 DECEMBER 1939

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**Kinetics of the thermal decomposition of fully deuterated diethyl ether.**  
By J. G. DAVOUD and C. N. HINSHELWOOD, F.R.S. (*Received 28 October 1939.*)

A detailed study of the kinetics of the decomposition of fully deuterated diethyl ether has been made. The results are discussed and compared with those (already published in the *Proceedings*) on the hydrogen ether.

---

**Rotational analysis of the first negative band spectrum of oxygen, II.**  
By T. E. NEVIN. (*Communicated by A. W. Conway, F.R.S.—Received 30 October 1939.*)

This is a continuation of work described in a previous paper on the structure of the first negative bands of oxygen. From an analysis of the (0, 0), (0, 1) and (1, 0) bands it was shown that the transition involved was  ${}^4\Sigma \rightarrow {}^4\Pi$ , the  ${}^4\Pi$  levels exhibiting an anomalous structure. The present paper gives an analysis of the (2, 0) and (0, 2) bands. This, in conjunction with the analysis in the previous paper, enables the vibrational constants of the system to be exactly calculated. The  ${}^4\Pi$  level  $v'' = 2$  shows the same structure as the previously analysed levels and an empirical formula is given which represents the separation of the components reasonably well. A complete table of the molecular constants for the five bands is given.

**On the evolution of the mammalian palate.** By F. R. PARRINGTON and T. S. WESTOLL. (*Communicated by J. Gray, F.R.S.—Received 31 October 1939.*)

The direct evidence of palaeontology shows that the Triassic Theriodonts possessed vomers and palatine processes directly comparable with those of mammals, and that these were developed from the reptilian prevomers and premaxillae respectively.

It is claimed that there is no evidence to show that the structures evolved in the Theriodont nose were ever changed.

It is shown that the fundamental assumptions on which the embryological evidence for the parasphenoid-vomer homology rest are quite untenable in the light of the resulting conclusions; or when the embryology of the *Chelonia* is taken into account; or when other structures besides the paraseptal cartilages are used as guides. The whole theory therefore rests on an entirely unreliable basis.

The embryological data can be interpreted more easily to fit the original "prevomer"-vomer homology.

The term "prevomer" is synonymous with vomer, and must lapse.

The homology of any mammalian pterygoid element with the lateral wing of the reptilian parasphenoid is similarly analysed and shown to be erroneous.

The "mammalian pterygoid" and "Echidna-ptyergoid" of monotremes are shown to be homologous respectively with the pterygoid and ectopterygoid of the Theriodonts and other reptiles.

The pterygoid of ditrematous mammals is found to include the homologue of the reptilian pterygoid; the ectopterygoid is occasionally represented separately, usually it is fused with the pterygoid. In some it may possibly be absent.

The fate of the reptilian parasphenoid in mammals is discussed, and it is concluded that the element is probably reduced altogether in normal mammals. Median splints present in certain forms are regarded as true parasphenoid vestiges.

The mutual relations of the palate and lower jaw are considered, with special reference to musculature and to changes in proportion.

**Properties of superconducting colloids and emulsions.** By D. SHOENBERG. (*Communicated by J. D. Cockcroft, F.R.S.—Received 31 October 1939.*)

Magnetization curves of colloids and emulsions of mercury are described which provide direct evidence for an appreciable penetration, increasing with temperature, of magnetic fields into small superconductors. From the temperature variation of  $\chi/\chi_0$ , the susceptibility ratio of a small to a large sphere, for a very fine colloid, the temperature dependence of the penetration depth  $\lambda$  is deduced, in fair agreement with the results of Appleyard and others. This is used to transform the temperature dependence of  $\chi/\chi_0$ , for an emulsion with  $r > \lambda$ , into a curve of  $\chi/\chi_0$  against  $\lambda/r$ , which is compared with a theoretical curve based on the London's penetration law, suggesting that the latter is only qualitatively valid. The critical fields  $h$  of the colloids and emulsions were larger than for bulk mercury, and it is shown how, together with the susceptibility data, they can be used to calculate the free energy difference per

unit volume between superconducting and normal phases for a small sphere. The results suggest that this difference increases (if at all) by a factor of at most 2 or 3, when the size is reduced from macroscopic dimensions to  $5 \times 10^{-6}$  cm. The shape of the magnetization curves and various hysteresis features are found to differ between a small and a large sphere, and these differences are discussed.

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**On the statistical method in nuclear theory.** By K. FUCHS. (*Communicated by M. Born, F.R.S.—Received 2 November 1939.*)

A generalization of the statistical method for the calculation of nuclear energies is given, which makes it possible to dispense with the Hartree approximation of independent wave functions.

The two directions of the spin are considered separately and general expressions for the energy of nuclei with any spin are derived.

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**Calculation of nuclear energies and stability by the statistical method.** By B. SPAIN. (*Communicated by M. Born, F.R.S.—Received 2 November 1939.*)

Nuclear energies and radii are calculated using the statistical method, taking into account the two possible spin directions; the interaction potentials of the various nuclear forces are assumed to be proportional to  $(k+a/r) \exp(-r/a)$ . The results obtained are in good agreement with experiment.

The stability of nuclei is also investigated, giving satisfactory agreement with experiment for odd nuclei, but for even nuclei the results are not so satisfactory. This seems to indicate that the statistical method does not emphasize sufficiently the difference between odd and even nuclei.

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**Some measurements of  $\gamma$ -ray energies.** By S. C. CURRAN, P. I. DEE and J. E. STROTHERS. (*Communicated by J. D. Cockcroft, F.R.S.—Received 2 November 1939.*)

Using the method of semicircular magnetic focusing of secondary electrons, it has been possible to construct an instrument which is suitable for the measurement of the quantum energies of  $\gamma$ -radiation between the limits of about 0.5 and  $16.0 \times 10^6$  eV, and which is capable of giving a reliable estimate of the relative intensities of the components of such radiation. The detecting system consists of three thin-walled Geiger-Müller counters coupled to an amplifying system which records triple coincidences. The construction of the apparatus is such that it may be used for the analysis of  $\gamma$ -radiation emitted during bombardment of light elements by protons as well as that emitted from radioactive sources.

The apparatus was standardized by using the  $\gamma$ -radiation from the active deposit

of thorium. Its accuracy having been thus established, it was used in the investigation of the  $\gamma$ -radiation from the sources  $^{24}\text{Na}$ ,  $^{56}\text{Mn}$ , and  $^{36}\text{Cl}$  and also the radiation emitted during the bombardment of fluorine by high energy protons. The results are tabulated below.

Source	Quantum energies	Relative intensities
	$\times 10^6 \text{ eV}$	
$^{24}\text{Na}$	1.46; 2.0; 3.03	1.17 : 0.27 : 1.0
$^{56}\text{Mn}$	0.91; 2.03	2.1 : 1.0
$^{36}\text{Cl}$	1.65; 2.15	3 : 4
$^{19}\text{F} + ^1\text{H} \rightarrow$	6.5	

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**The  $\beta$ -ray spectrum of radium E.** By G. J. NEARY. (*Communicated by J. D. Cockcroft, F.R.S.—Received 2 November 1939.*)

The  $\beta$ -ray spectrum of Ra E has been investigated with a magnetic spectrograph of special design with a source on  $\frac{1}{2}\mu$  aluminium and a counter window of  $10^{-3} \text{ g./cm.}^2$  Zaponlak.

It is considered that the experimental results represent directly the distribution of particles from about 20 keV upwards without distortion from any cause. The energy curve has a maximum at 150 keV and an end-point at 1.17 MeV, with a mean energy of 340 keV, in complete agreement with calorimetric determinations. The momentum curve has a maximum at  $H\rho = 1800 \text{ G-cm.}$  in satisfactory agreement with Flammersfeld,  $H\rho = 1750 \text{ G-cm.}$  The intermediate part of the K.U. plot is straight, the energy difference between experimental and extrapolated end-points being  $0.55 m_0 c^2$ ; on the other hand, the introduction of a finite neutrino mass does not bring about agreement with theory. The K.U. plot also falls below the straight line below 150 keV, and resembles some of the possible distributions for "forbidden" transitions, as given by Hoyle.

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**The oviposition behaviour of parasites of *Plutella maculipennis* Curt.** By D. C. LLOYD. (*Communicated by W. R. Thompson, F.R.S.—Received 8 November 1939.*)

An investigation is being made of the incidence in the laboratory and field of superparasitism and multiparasitism in the primary parasites of *Plutella maculipennis* Curt. The present paper deals mainly with laboratory experimental work on the three species—*Diadromus collaris* Grav., *Angitia cerophaga* Grav., and *Apanteles plutellae* Kurdj.

From the experimental point of view, the problem of multiple parasitism is being regarded as a question of the nature of the oviposition responses of the various

parasites. The procedure adopted is to confine the female parasites in tubes for a fixed time with a given number of hosts and to observe the oviposition behaviour of the adult parasites, and in the subsequent development of the immature parasites to note the effects of competition.

With *Diadromus collaris* the silken cocoon surrounding the pupa is important in securing maximum oviposition because of the posture adopted by the female in the oviposition act. Unparasitized hosts were more acceptable when histogenesis was in the early stages, but hosts parasitized in such stages were usually rejected by subsequent females. This rejection was very well defined in respect of pupae parasitized by *D. collaris* itself, or prepupae containing advanced stages of *Angitia cerophaga*, and was exercised when such hosts were given to the females for numerous brief exposures. Superparasitism in pupae and prepupae occurred only in cases where the host contained recently deposited eggs; multiparasitism obtained when the *Diadromus* female encountered prepupae containing early instars of *Angitia cerophaga*, but oviposition in these conditions was restricted. Experiments suggest that feeding in host contents is necessary for the full development of the gonads.

The parasite *A. cerophaga* will attack all host larval instars and also occasionally prepupae in cocoons. Larvae containing eggs of first and second instars of *A. cerophaga* were usually rejected when unparasitized hosts were available; when parasitized hosts only were given to the females, oviposition was limited. An ability to distinguish between unparasitized host larvae and those containing eggs of *Apanteles phutellae* was also observed.

*A. phutellae* oviposited in the first three host larval instars and tended to avoid parasitizing hosts containing immature stages of its own species or those of *Angitia cerophaga*.

Elimination of supernumerary larvae in superparasitism in *Diadromus collaris* was usually by combat in the first and second instars. When present in a prepupa with an early instar of *Angitia cerophaga* neither species survived. Suppression of excess larvae in superparasitism in *A. cerophaga* and *Apanteles phutellae* was by physiological inhibition in the first instar. This also occurred when specimens of both species were found in one host, but neither species appeared intrinsically superior if the eggs were laid within a short interval.

Some data on field parasitism in the first generation of the host in North Holland in 1939 are included. The details for superparasitism and multiparasitism support laboratory experimental work.

The relation of the findings to biological control practice of introducing several primary parasites of a given pest is briefly examined.

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Tables for elucidating the internuclear vibrations of molecules and crystals. By A. C. MENZIES and K. WEISSENBERG. (Communicated by C. G. Darwin, F.R.S.—Received 9 November 1939.)

Tables of symmetry properties (including activity in the Raman effect and infra-red) are described which apply to the internuclear oscillations of molecules, and to the

"homogeneous" or "lattice" oscillations of crystals. An explanation is given of the use of the tables for separating the oscillations of crystals into "internal" and "external" ones, and, in the case of the latter, into oscillations of a translational and of a rotational type.

The fundamental normal modes are characterized by a numerical notation in place of the various alphabetic ones in use; this notation consists of sets of numbers ("mode numbers") which are a condensed form of the representation of the fundamental mode in the symmetry group. This allows the symmetry properties of combinations to be calculated in a very simple way, and the rules for doing this are given. The overtones up to the sixth are tabulated explicitly.

At every stage examples are given to show how the tables can be used, and finally the oscillations of the crystals of urea and ammonium nitrate are considered in detail.

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**On the hardening of the cuticle in the ootheca of *Blatta orientalis*.**  
By M. G. M. PRYOR. (*Communicated by A. D. Imms, F.R.S.—Received 13 November 1939.*)

The series of reactions concerned in the hardening and darkening of the ootheca of *Blatta orientalis* have been studied in the hope that they may throw some light on the hardening of the cuticle of insects in general. The ootheca is secreted by two glands, of which one secretes a water soluble protein, and the other a dihydroxyphenol. After secretion the phenol is oxidized, probably by an enzyme, to the corresponding quinone, which then combines with the protein by a reaction similar to that involved in the tanning of collagen by benzoquinone. This results in the introduction of primary valence cross linkages into the protein network, so that it becomes rigid and very resistant to all chemical reagents and enzymes. The protein of the mature ootheca is more stable than keratin, and represents a new type of scleroprotein, for which the name "sclerotin" is proposed.

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**On the hardening of the cuticle of insects.** By M. G. M. PRYOR. (*Communicated by A. D. Imms, F.R.S.—Received 13 November 1939.*)

The hardening of the insect cuticle is due to the formation of a protein similar to that of the cockroach ootheca. A water soluble protein and dihydroxyphenol are secreted into the outer layers of the cuticle, and oxidation products of the phenol there react with the protein to form sclerotin. A similar reaction takes place in the epicuticle, which is secreted as a protein membrane and is subsequently "tanned" by the introduction of aromatic cross linkages. After it has been converted into sclerotin in this way, the epicuticle is impregnated with lipids, which oxidize and



polymerize until they become insoluble in fat solvents. The cuticle of the Crustacea is like the epicuticle of insects in chemical composition, but differs in that the lipids are less highly polymerized.

The phenol which takes part in the reaction to form sclerotin is probably dihydroxyphenyl-acetic acid, or some very similar compound: the phenol in the blood, which is responsible for melanosis after death or at the site of injuries, will not react with proteins *in vitro*, and so cannot be directly concerned in the formation of sclerotin. The blood phenol appears to be dihydroxyphenyl-alanine, which is transported in the blood from some organ in which it is synthesized to the cells of the hypodermis, where it is de-aminated, and converted to the phenolic component of sclerotin.

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**On the natural frequencies of vibrating systems.** By R. V. SOUTHWELL, F.R.S. (*Received 15 November 1939.*)

On the basis of a theorem due to Lord Rayleigh and relating to the effect on the natural frequencies of an added mass, methods are developed whereby lower limits can be imposed upon the frequencies of a specified system. Since upper limits can be imposed on the basis of "Rayleigh's principle", information so obtained is for practical purposes of equal value with an exact solution.

The methods can be applied as an extension of the "relaxation" technique, and it is then that their value is revealed most clearly. In this paper attention is confined to continuous systems governed by differential equations, and for these, incidentally, a method is developed whereby specially close estimates of the fundamental frequency can be made if desired.

The concluding section of the paper is concerned with the resolution of a paradox presented by Lord Rayleigh's theorem regarding the effect of a constraint.

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**Hyperfine structure in the arc spectrum of bromine.** By S. TOLANSKY and S. A. TRIVEDI. (*Communicated by W. E. Curtis, F.R.S.—Received 21 November 1939.*)

Sixty-five classified lines of the Br I spectrum, lying in the region 8650–4350 Å, have been investigated for hyperfine structure and analysed. It is confirmed that the nuclear mechanical and magnetic moments of both isotopes are the same. No isotope displacement is observed. Hyperfine structure interval factors are derived for thirty-eight terms amongst which are seven complete multiplet groups. From the

hyperfine structure analysis it has been possible to correct some errors in the multiplet analysis.

The coupling of the  $4p^4$  electron group with the nucleus is shown to be quite large. This group therefore behaves in a manner similar to that of the corresponding  $5p^4$  group of iodine.

The  $4s^2 4p^4 5s^2 P_1$  term exhibits deviation from the interval rule and this accurately obeys the quadratic formula for the interaction which arises when the nucleus has an electrical quadrupole moment. The interaction formula for this term is

$$E = 223 + \frac{1}{4} \cdot 47 \cdot 1C + 0 \cdot 17C(C+1) \text{ cm.}^{-1} \times 10^{-3}.$$

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